THE AMERICAN JOURNAL OF Clinical Nutrition

MAY–JUNE 1958 VOLUME 6, NUMBER 3

Symposium on

The Mode of Action of Lipotropic Factors in Nutrition*

THE GRADUATE School of Public Health of the University of Pittsburgh takes pleasure in welcoming all of the participants in this *Symposiium on The Mode of Action* of Lipotropic Factors in Nutrition to our campus and to our city. As most of you know, we are indebted to The National Vitamin Foundation for joint sponsorship of this Symposium, that 51 per cent of sponsorship in fact, which has provided the wherewithal to permit us to gather here today to take stock of our knowledge in this important field. I'm sure that all of you join me in paying my compliments to Dr. Robert S. Goodhart, the Scientific Director of The National Vitamin Foundation, who has been instrumental in the organization of at least two such Symposia per year for the past several years. These Symposia have provided a meeting place for the basic scientist and the clinician working in various fields of nutrition to appraise current research and exchange views. Experimental and clinical nutrition are fields in which the workers not only profit from better communication between their respective groups but also gain enrichment from intimate contact with the respective basic and clinical disciplines which underlie them. This cross fertilization is one of the dividends which has emerged from these Symposia in the past and I am hopeful that such dividends may be forthcoming from this Symposium as well.

With regard to the subject under discussion, *the mode of action of lipotropic factors*, we might recall that the field was opened by the discovery of Allan *et al.*[1] that depancreatized dogs maintained with insulin developed fatty livers. Subsequently, Best and Hershey[2] showed that raw pancreas supplements to the diet of depancreatized dogs protected against fatty liver and that the active principle was lecithin. The production of "dietary fatty liver" in the rat by Best and Huntsman[3] and the demonstration that choline was the active lipotropic agent in lecithin, opened the way for hundreds of productive investigations of the role of specific dietary nutrients in the control of fat content of the liver in this species. It is somewhat ironic that although the replacement of pancreas in the diet of the insulin-treated depancreatized dog prevented the fatty liver which ordinarily developed, it did so for a different reason than that of the intact functioning pancreas. As more recent studies have shown,

* Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc., New York, New York.

the intact pancreas supplied proteolytic enzymes essential for the liberation of *methionine* from dietary protein[4] whereas the dietary pancreatic tissue supplied *choline* which produced the same hepatic effect. An explanation for the sometimes clear,[5] sometimes enigmatic, interrelationship of choline and methionine in influencing liver lipid content, lipid transport, kidney structure and function and even vascular integrity will most certainly be the concern of many of the essayists in this Symposium.

The term lipotropic is derived from the Greek *lipos* meaning "fat" and the Greek *trope* meaning "turning." Literally, it means the "turning of fat." It was first used by Best, Huntsman, and Ridout in one of their early papers.[6] As originally defined "lipotropic" compounds were those substances which "decreased the rate of deposition or accelerated the rate of removal of liver fat." Through common usage, the term has been applied to the action of substances upon histologically visible fat in any tissue which ordinarily does not have visible fat present. I am hopeful that we shall return, in this Symposium, to a more literal use of the term. I suggest that we think in terms of the relationship of this group of nutrients to the "turnover of lipid" in the dynamic sense—considering their role in various processes by which lipid is synthesized, enters, becomes visible, is oxidized or leaves the organ under consideration.

This Symposium was planned to embody a dual progression: from enzymology to physiology—and from animal to man. This morning Dr. Stekol will tell us about the biosynthesis of "labile" methyl groups, transmethylation and the biosynthesis of choline. Dr. Kennedy will next review his important contributions dealing with the enzymatic synthesis of phospholipid from choline. Next, Dr. Artom will review for us the evidence that lipotropic factors play a role in the oxidation of fatty acids and Dr. Zilversmit will present data on the role of lipotropic factors in the turnover of phospholipids.

We shall then turn to animal nutrition for a consideration by Dr. Harper of the dietary factors which influence liver fat deposition in the rat and a discussion by Dr. Wells of the effects in the animal of antimetabolites to the lipotropic factors. Dr. Griffith will review his classical work on the renal lesion in choline deficiency and Dr. Wilgram will define the role of the lipotropic factors in maintaining the integrity of the heart and vascular tree in the rat.

We then plan to take a great leap from the experimental to the clinical situation and consider the information which is available about fatty liver in man. Dr. Frenk will discuss kwashiorkor and marasmus in children and Dr. Gabuzda fatty liver in adults. Next, Dr. Labecki and I shall contemplate the evidence which ascribes a role to these factors in controlling the blood lipids in man.

When the last word is said, it is doubtful that we shall have answered the question posed by this Symposium in entirety. If some questions are answered, if a clearer definition of the problem is obtained, if ideas about new approaches to the problem are sparked from the discussions, then this meeting will be well worth while. Further research is essential to answer many questions, particularly those dealing with the relevance of the results of animal experimentation to man. The etiology and pathogenesis of fatty liver in the human are still not known with certainty. Further, the relationship of liver function in man to the pathogenesis of atherosclerosis in states of under- and overnutrition is a field which demands further thorough and systematic exploration.

At present, however, unprecedented opportunities exist for the study of liver function and metabolism in man. Safe procedures for liver biopsy, micromethods for enzyme assay, access to hepatic venous blood via cardiac catheters, isotopic methods for the study of intermediary metabolism, and knowledge to achieve highly specific dietary control are realities and should provide an exciting challenge to the clinical investigator working in this field.

Finally, since research and teaching go hand in hand in most medical centers, it seems to me that the field of clinical nutrition provides an avenue for the teaching of "comprehensive

medicine" which is not available in many other fields of medicine. The recent emphasis upon consideration of the "total man" in modern medical schools has meant improved coverage in breadth—i.e., of the social and behavioral aspects of the clinical problem in addition to the conventional study of the medical history, physical examination, and the common laboratory parameters. I should like to suggest that there is another dimension which should be covered in the approach to the "total man" and that is the vertical plane via which one uncovers the processes of cellular physiology which underlie the clinical syndrome. The field of nutrition not only has its behavioral overtones but is undergirded with a wealth of information about the function of the nutrients in cellular physiology and their relation to intermediary metabolism. I hope that the range of subject matter embraced by this Symposium—from the origin of the C_1-fragment to the pathogenesis of fatty liver in the chronic alcoholic will provide a panoramic view of what the field of nutrition has to offer to both the advanced researcher and to the student of medicine.

—Robert E. Olson, ph.d., m.d.
Dept. of Biochemistry and Nutrition
Graduate School of Public Health
University of Pittsburgh

REFERENCES

1. Allan, F. N., Bowie, D. J., MacLeod, J. J. R., and Robinson, W. L. Behaviour of depancreatized dogs kept alive with insulin. *Brit. J. Exper. Path.* 5: 75, 1924.
2. Best, C. H. and Hershey, J. M.: Further observations on the effects of some components of crude lecithine on depancreatized animals. *J. Physiol.* 75: 49, 1932.
3. Best, C. H. and Huntsman, E.: The effects of the components of lecithine upon deposition of fat in the liver. *J. Physiol.* 75: 405, 1932.
4. Chaikoff, I. L., Entenman, C., and Montgomery, M. L.: The mechanisms of action of the antifatty liver factor of the pancreas. *J. Biol. Chem.* 160: 489, 1945.
5. Du Vigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B.: The transfer of the methyl group from methionine to choline and creatine. *J. Biol. Chem.* 134: 787, 1940.
6. Best, C. H., Huntsman, M. E., and Ridout, J. H.: The lipotropic effect of protein. *Nature* 135: 821, 1935.

Biosynthesis of Choline and Betaine

JAKOB A. STEKOL, M.A., SC.D.*

THE PREVALENT hypothesis of the biosynthesis of choline states that ethanolamine is methylated *in vivo* by the stepwise transfer of the methyl group of methionine. This hypothesis was outlined by Jukes[1] and Artom[2] in a schema which is shown in Figure 1. The ethanolamine moiety of choline is formed from serine by decarboxylation. Ethanolamine is then presumed to be methylated in a stepwise fashion by the specifically activated methyl donor, namely "active methionine," to give successively, methylaminoethanol, dimethylaminoethanol, and finally choline.

We shall review briefly the experimental data upon which this schema was based, and later we shall present additional information which does not seem to fit into this schema.

SYNTHESIS OF ETHANOLAMINE FROM SERINE

Stetten[3] demonstrated the appearance of N^{15} of glycine-N^{15} and serine-N^{15} in the ethanolamine moiety of tissue choline. The formation of ethanolamine from glycine proceeds via the intermediate formation of serine, followed by the decarboxylation of the latter. Levine and Tarver[4] have shown that in animals receiving serine-3-C^{14} the isotope appeared in tissue ethanolamine, while none of the isotope of glycine-1-C^{14} was found in the tissue ethanolamine.[5] It is presumed that the enzymatic system which is involved in the decarboxylation of serine to ethanolamine is a pyridoxal phosphate-dependent system. According to this assumption, choline synthesis in the pyridoxine-deficient animals should be impaired. This was found to be the case in intact pyridoxine-deficient rats,[6] suggesting that an impediment in the decarboxylation of serine to ethanolamine in this deficiency impairs the utilization of the methyl group of methionine for choline biosynthesis.

HYPOTHESIS OF STEPWISE METHYLATION OF ETHANOLAMINE

The production of fatty livers in rats which were maintained on diets deficient in choline,[7] and the alleviation of the fatty infiltration of the liver by diets high in protein or methioine[8] could be considered the major observations upon which all subsequent developments in regard to the mode of choline biosynthesis have been based. The first clear-cut theory, based on nutritional and biochemical evidence, was proposed by du Vigneaud and associates[9] in which the new concept of transmethylation was announced. This concept, elaborated and corroborated over a span of years, stated that the methyl groups of choline originate *in vivo* via a direct transfer of the intact methyl group of methionine to an acceptor, ethanolamine, and that this process is reversible, i.e., the methyl groups of choline are the source of the methyl group of methionine *in vivo* on diets containing homocystine.[10] This biologically migrating group was named the "labile methyl group," and the process of its migration *in vivo* was named "transmethylation." On the basis of evidence available at that time it was further stressed that this labile methyl group is indispensable in the diet of animals. The evidence for this assertion overwhelmingly favored it, the only disagreement being whether to classify choline as a vitamin, along with the

From The Lankenau Hospital Research Institute and the Institute for Cancer Research, Philadelphia, Pa.

* Head of Department of Physiological Chemistry and Nutrition, The Lankenau Hospital Research Institute and the Institute for Cancer Research, Philadelphia, Pa.

This paper was presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition*, held at the University of Pittsburgh on October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc., New York, N. Y.

The author wishes to acknowledge the assistance through grants by the U S Atomic Energy Commission, the National Cancer Institute, National Institutes of Health, Bethesda, Maryland, and through an Institutional Grant of the American Cancer Society.

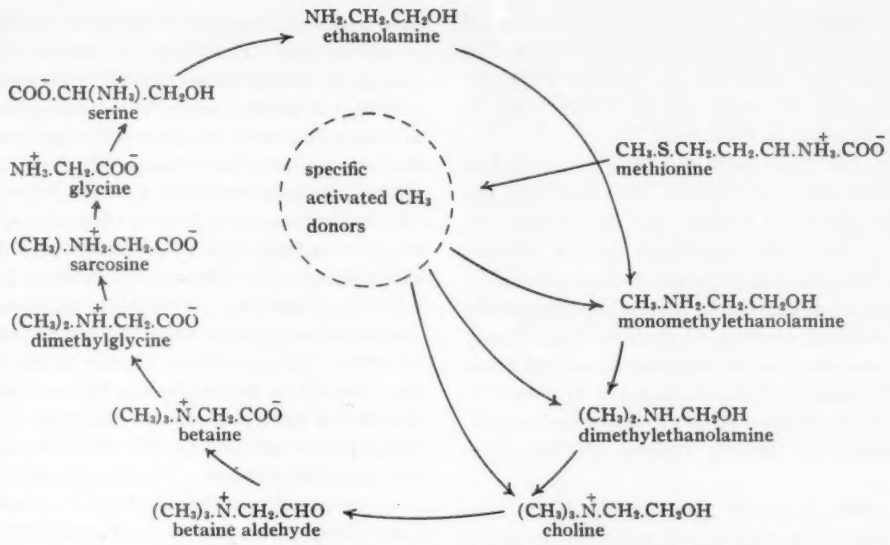


Fig. 1. A schema for the stepwise methylation of ethanolamine by methyl of methionine via transmethylation.

status of a "labile methyl donor," or as an indispensable lipotropic nutrient. This somewhat teleologic argument, unsettled as yet in some textbooks, did not, however, affect the consensus that choline is indispensable in the diet of animals, unless sufficient methionine was present in the diet to cover adequately the need for growth and biosynthesis of choline via transmethylation. The labile methyl group itself, however, was ruled unsynthesizable *de novo*.

The chick, on the other hand, exhibited an absolute requirement for choline in the diet,[11] unless the diet contained dimethylaminoethanol as well as methionine.[12] Ethanolamine or methylaminoethanol, in lieu of dimethylaminoethanol, as the acceptor of the methyl of methionine, would not satisfy the chick. These and similar data on guinea pigs[13] indicated that these species are unable to synthesize dimethylaminoethanol from ethanolamine or methylaminoethanol and methionine, and that a genetic block existed in the guinea pig and in the chick which prevented the methylation of ethanolamine by methionine to give choline. Horowitz[14] observed that in certain mutants of *Neurospora* genetic biochemical blocks exist which prevent the biosynthesis of methylaminoethanol or dimethylaminoethanol from ethanolamine and methylaminoethanol respectively. These observations together with those on the chicks, described above, furnished the main basic facts upon which the theory of stepwise methylation of ethanolamine via transmethylation from methionine was formulated. The important point to note is that although the *Neurospora* experiments revealed a stepwise *formation* of the methyl groups of choline, no evidence was provided in these experiments that *such a stepwise formation of the methyl groups of choline proceeded in the mold via the process of transmethylation from methionine.* These experiments on *Neurospora* only indicated that the stepwise *formation* of the methyl groups of choline is substrate-specific, being a function of genetically controlled and enzymatically distinct reactions. In spite of these implications it was generally assumed that the sole source of the methyl groups of choline was methionine.

It should be pointed out, however, that at the time no other conclusion was feasible, because the *de novo* synthesis of the labile methyl group was unacceptable, and the available evidence for the *de novo* synthesis of the labile methyl group was attributed to the activity of

the intestinal flora. As late as 1949 it was still asserted by the principal investigators in this field that the labile methyl groups of choline and methionine could not be synthesized *de novo* by the animal organism.

With the experimental evidence provided in 1949 and since that time to the effect that the methyl groups of choline and methionine can arise *de novo* from numerous dietary components, other than methionine, choline, or betaine, it became imperative to review experimentally the prevalent concept of choline biosynthesis. This was particularly desirable in view of some evidence which indicated that the *de novo* synthesis of the labile methyl group depends on the availability of dietary vitamin B_{12} and folic acid.

As often happens when newly discovered facts emerge in regard to a generally accepted hypothesis, a certain amount of confusion developed around the theory of transmethylation and its role in choline biosynthesis. If the methyl groups of choline depend in their neosynthesis on vitamin B_{12}, how then can one reconcile this fact with the formation of choline from methionine on diets which were apparently free of this vitamin? Is the methyl of methionine still the sole source of the methyls of choline, or can the neosynthesized methyls of choline arise via some intermediate other than methionine? Is it possible then that the methyl of methionine arose *de novo* via the intermediate formation of some other metabolite? Or are we to assume that regardless of the nature of the compound which carries a N-methyl, S-methyl, or C-methyl group, which was synthesized *de novo*, this methyl group arose first in methionine and then was transferred from methionine by the process of transmethylation? Thus, the N-methyl, S-methyl, and C-methyl groups would have a common origin, namely methionine. Such a view seems to have found favor with some reviewers of the subject.[15] There are some facts, however, that do not seem to fit into this generalization. For instance, the methyl group of thymine is readily synthesized from formate or serine-3-C^{14}, but not from methionine.[16] However, 5-hydroxymethylcytosine gave rise to thymine.[17] As we shall see presently, in the folic acid-deficient rats the methyl of methionine is not readily transferred to either ethanolamine or methylaminoethanol to give choline, although folic acid, or its biological equivalent, is not a cofactor in the process of transmethylation to or from methionine.[6,18] Noland and Baumann[19] reported that roaches fail to grow or survive on a diet free of choline. Choline may be substituted by betaine, but not by ethanolamine, methionine, or dimethylaminoethanol. However, methionine and dimethylaminoethanol, given together, were partially effective. According to Artom,[2] "it would appear, therefore, that at least in these insects the transfer of methyl groups to ethanolamine and methylamino-ethanol occurs from betaine but not from methionine. It may be noted that previous results on the synthesis of choline by liver tissue incubated with ethanolamine and methionine[20] have been questioned recently.[21] These and other considerations led Artom[2] to remark that "as for stepwise methylation of ethanolamine to choline, it should be pointed out that evidence for such a process is still quite indirect and perhaps susceptible of somewhat different interpretation." Considerations of a laboratory synthesis of dimethylaminoethanol from methylaminoethanol and formaldehyde led Mackenzie[22] to speculate about the possibility that in the *in vivo* synthesis of choline only one methyl of methionine is transferred to an acceptor, while the other two methyls of the acceptor could possibly arise *in vivo* by a process analogous to that employed in the laboratory synthesis of dimethylaminoethanol.

The facts and considerations cited above should not be construed as evidence against the well substantiated theory of transmethylation, namely, the transfer of an intact methyl group of methionine in certain biochemically distinct and enzymatically definable reactions. Some of such enzymatically definable reactions which are involved in the biosynthesis of choline, creatine, N′-methylnicotinamide, etc., almost certainly proceed via the process of transmethylation. The careful experiments of du Vigneaud and associates, employing intramolecularly-labeled methionine (C^{14} and deuterium), demonstrate, within the limits of ac-

curacy of the analytical methods used, that whenever such a transfer of the methyl of methionine *in vivo* takes place, the group migrates to an acceptor intact, without any measurable alteration in the ratio of its carbon to hydrogen. These experiments, however, do not prove, and they were not designed to prove, that in the case of the biosynthesis of choline *all three methyl groups of choline arose in vivo via transmethylation from methionine*, or that such migration of intact methyls of methionine took place via the above mentioned stepwise methylation, illustrated in Figure 1. The chemical analytic procedure employed in the degradation of the isolated choline involves the formation of trimethylamine which is then assayed for its total isotope content. This procedure precludes the possibility of isotope assay *in each of the three methyl groups of choline* in order to find out whether the isotope of the trimethylamine moiety of choline is evenly distributed among the three methyl groups, as is generally assumed. Had only one, two, or all three methyl groups of choline originated from methionine via transmethylation, the analytical results for the ratio of C^{14} to deuterium in the methyls of choline would have been the same.[23]

The problem before us, therefore, is to ascertain whether the process of transmethylation from methionine to choline is the exclusive process in the biosynthesis of choline, or whether the process of transmethylation from methionine is only one of the essential steps in the synthesis of its methyl groups. As can be readily seen, posing the problem of biosynthesis of choline in this way does not in any way exclude the participation of the process of transmethylation from methionine to choline. It merely attempts to define the place for the process of transmethylation in the sequence of reactions which at the same time with transmethylation generate *de novo* the methyl groups of choline.

We shall now proceed with the presentation of data bearing on this problem, obtained in our laboratory with the cooperation of Drs. S. Weiss, E. I. Anderson, Peng Tung Hsu, and M. Toporek, and Miss P. Smith, Miss A. Watjen, and Miss K. W. Weiss.

VITAMIN B_{12} AND FOLIC ACID IN TRANSMETHYLATION REACTIONS

It appeared to us essential to establish first whether vitamin B_{12} and folic acid, or their biologic equivalents, play a role in transmethylation reactions to or from methionine. In these studies we employed rats, chicks, and several strains of mice. The data, briefly summarized in Table I, show that the deficiency in vitamin B_{12} in these animals has no measurable effect on the extent of incorporation of the radiomethyl group of methionine into the phospholipid choline.[6]

TABLE I

Vitamin B_{12} in the Synthesis of Choline from Methionine-CH_3-C^{14}

Animal	Standard specific activity of choline × 100†	
	Normal	Vitamin B_{12}-deficient
Rat	35.0	47.0
Mouse	30.0	31.6
Chick*	21.0	17.3

* The diet contained homocystine and dimethylaminoethanol. Choline was isolated from the entire carcass 4 hours (mice) and 20 hours (rats and chicks) after the injection of radiomethylmethionine.

† "Standard Specific Activity" is the relative specific activity of the isolated metabolite divided by the dose of the radioisotope injected in mmol/100 g weight of the animal.

The data in Table II show that the deficiency in vitamin B_{12} also had no measurable effect on the extent of incorporation of the C^{14} of the methyl groups of choline or betaine into tissue methionine.[18] These results were not in accord with those of Ericson, *et al.*[24] who claimed to

TABLE II

Vitamin B_{12} in the Synthesis of Methionine from Choline-CH_3-C^{14} and Betaine-CH_3-C^{14}

Animal	Isotope injected*	Standard specific activity of methionine × 100	
		Normal	Vitamin B_{12}-deficient
Rat	Betaine-CH_3-C^{14}	2.56	2.91
"	Choline-CH_3-C^{14}	0.26	0.28
Chick	Betaine-CH_3-C^{14}	7.40	7.20
"	Choline-CH_3-C^{14}	2.12	2.20
Mouse	Betaine-CH_3-C^{14}	3.22	3.24
"	Choline-CH_3-C^{14}	0.33	0.38

* Methionine was isolated from the entire carcass 4 hours (mice) and 20 hours (chicks and rats) after the injection of the isotopic compounds.

TABLE III

Folic Acid in the Synthesis of Methionine from Betaine-CH_3-C^{14}

Animal*	Standard specific activity of methionine × 100	
	Normal	Folic acid-deficient
Rat	2.56	3.00
Mouse	3.22	3.10
Chick	7.40	7.60

* Methionine was isolated from the entire carcass 4 hours (mouse) and 20 hours (rats and chicks) after the injection of radiobetaine.

have observed a reduction in the activity of betaine transmethylase in the livers of vitamin B_{12}-deficient rats. These workers also claimed to have resolved betaine transmethylase into an apoenzyme and a cofactor fraction, although the cofactor fraction proved to be free of vitamin B_{12} activity.[25] The resolution of betaine transmethylase into the cofactor and an apoenzyme fraction could not, however, be confirmed.[26]

The data in Table III show that the extent of incorporation of the radiomethyl group of betaine into tissue methionine in rats, mice, and chicks is not inhibited by the deficiency in folic acid. On the basis of these data and those shown in Tables I and II we concluded that neither folic acid nor vitamin B_{12} is apparently involved in the *in vivo* transfer of the methyl group to or from methionine in the rat, the mouse, or the chick.[18]

We next examined the extent of utilization of the radiomethyl group of methionine and betaine in the synthesis of choline in folic acid-deficient animals. It has already been known that the deficiency in folic acid produces severe fatty infiltration in the livers of rats, suggesting a limited synthesis of choline in this deficiency.[27] The data in Table IV show a marked inhibition of the extent of incorporation of the radiomethyl groups of methionine and betaine into the phospholipid choline in animals which were deficient in folic acid. Since, as has been shown in Table III, the synthesis of methionine from betaine was not inhibited by the deficiency of folic acid, it was apparent that the inhibition of choline biosynthesis from betaine was a result of some aberration in the utilization of methionine that was formed from betaine. Previous data excluded the participation of folic acid or of its biologic equivalents in the transmethylation reactions involving methionine, and the biosynthesis of ethanolamine from serine does not depend on folic acid as the cofactor in the decarboxylation of serine. We, therefore, interpreted the observed inhibition of the incorporation of the radiomethyl group of methionine into choline by the deficiency in folic acid as being due to an inhibition of biosynthesis of the direct acceptor of the methyl group of methionine.[6] As already stated, this direct acceptor of the methyl of methionine could not have been ethanolamine, since its formation does not depend on folic acid or its derivatives. It was, however, possible that the deficiency in folic acid produces an inhibition in choline biosynthesis from methionine indirectly, possibly via an inhibition of synthesis of factors or cofactors which are directly concerned with the transfer of the methyl of methionine to choline. It should be noted at this point that the deficiency in vitamin B_{12} or in folic acid in these animals had no measurable effects on the extent of incorporation of choline into tissue phospholipids, ruling out the possibility that the deficiency in folic acid curtailed the synthesis of a factor or cofactor which is concerned in the formation of phospholipids from choline. This was an important point to establish, because in all our studies on the biosynthesis of choline phospholipid choline was the material investigated.

In the following experiments, frankly of exploratory nature, we set out to ascertain the possible reasons for the inhibition of the utiliza-

TABLE IV

Folic Acid in the Synthesis of Choline from Methionine-CH_3-C^{14} and Betaine-CH_3-^{14}

Animal	Isotope injected*	Standard specific activity of choline × 100	
		Normal	Folic acid-deficient
Rat	Methionine-CH_3-C^{14}	35.0	14.0
"	Betaine-CH_3-C^{14}	26.0	17.0
Mouse	Methionine-CH_3-C^{14}	30.0	19.0
"	Betaine-CH_3-C^{14}	19.0	12.0

* Choline was isolated from the entire carcass 4 hours (mice) and 20 hours (rats) after the injection of isotopes.

tion of the radiomethyl group of methionine for choline formation in the folic acid-deficient animals, and to explore the possible nature of the direct acceptor of the methyl of methionine, the biosynthesis of which in the folic acid deficient animals, we believed, was inhibited.

Employing the folic acid-deficient rats, we next examined the effects of administration to these animals of ethanolamine, methylaminoethanol or dimethylaminoethanol on the extent of biosynthesis of choline from radiomethylmethionine. The data in Table V show that the simultaneous administration of ethanolamine or methylaminoethanol and radiomethylmethionine to folic acid-deficient rats did not improve the biosynthesis of choline. On the other hand, the simultaneous administration of dimethylaminoethanol or of the *Citrovorum factor* and radiomethylmethionine to the same rats promptly increased the extent of choline formation. These data suggested that the deficiency in folic acid in rats inhibited the biosynthesis of dimethylaminoethanol, and that the latter is the direct acceptor of the methyl group of methionine *in vivo*. Folic acid or its biological equivalent, however, is not involved in the transfer of the methyl group of methionine to dimethylaminoethanol.[28] Previous studies by du Vigneaud and associates,[29] employing deuterium-labeled dimethylaminoethanol, indicated an efficient synthesis of tissue choline in normal rats from dimethylaminoethanol, the extent of incorporation of the labeled dimethylaminoethanol into tissue choline being of the order of 80 per cent of the extent of incorporation of deuteriomethyl-labeled choline into tissue choline.

TABLE V

Folic Acid in the Synthesis of Phospholipid Choline from Choline-CH_3-C^{14} and Methionine-CH_3-C^{14}

Isotope injected*	Nonisotopic supplement injected with isotope	Standard specific activity of choline × 100	
		Normal	Folic acid-deficient
Choline-CH_3-C^{14}	None	73.0	76.0
Methionine-CH_3-C^{14}	None	30.0	18.4
"	Aminoethanol	—	17.8
"	Methylaminoethanol	—	20.0
"	Dimethylaminoethanol	—	38.8
"	*Citrovorum factor*	29.8	34.8

* 0.003 mmol of isotope material per 100 g weight of the rat alone or together with 0.1 mmol of the nonisotopic methionines were injected intraperitoneally in a single dose. 0.1 mg. of *Citrovorum factor* (Leucovorin, Lederle) was injected 2 hr before the injection of the radiomethionine. All animals were sacrificed 1 hour after the injection of the isotopes, and choline was isolated from the entire carcasses.

It occurred to us that if the deficiency in folic acid inhibits the biosynthesis of dimethylaminoethanol from ethanolamine, then the extent of incorporation of the C^{14}-labeled ethanolamine into tissue choline in folic acid-deficient rats, supplied with ample unlabeled methionine, should be lower than in normal animals. The data in Table VI show that the extent of incorporation of the labeled ethanolamine into tissue choline of folic acid-deficient rats was markedly lower than in normal animals. On the other hand, the extent of utilization of radiomethyl-dimethylaminoethanol for the biosynthesis of choline was not affected by the deficiency in folic acid.[28]

TABLE VI

Utilization of Ethanolamine-1,2-C^{14} and Dimethylaminoethanol-CH_3-C^{14} by Normal and Folic Acid-Deficient Rats for Choline Synthesis

Isotope injected*	Hours after injection of the isotopes	Standard specific activity of	
		Normal rat	Deficient rat
Ethanolamine-1,2-C^{14}	1	3.0	0.7
	20	32.0	12.0
Dimethylaminoethanol-CH_3-C^{14}	1	40.0	41.0
	20	62.0	65.0

* 0.003 mmol of the isotopic material per 100 g weight of the rat together with 10 g of nonisotopic methionine were injected intraperitoneally in a single dose. Choline was isolated from the entire carcasses after the stipulated periods.

We next examined the extent of biosynthesis of tissue choline from methionine in normal and folic acid-deficient rats which received a continuous supply of ethanolamine, methylaminoethanol, dimethylaminoethanol, or choline in the diet. The data in Table VII show that the presence of ethanolamine,

TABLE VII

Utilization of Methionine-CH_3-C^{14} for Choline Synthesis by Normal Rats Which Received in the Diet a Continuous Supply of Ethanolamine, Methylaminoethanol, or Dimethylaminoethanol, or Choline

Supplement fed with diet %	Standard specific activity of choline × 100
None	35.0
Ethanolamine, 0.34	35.0
Methylaminoethanol, 0.40	32.0
Dimethylaminoethanol, 0.45	39.0
Choline, 0.50	24.0

Choline was isolated from the entire carcasses 20 hours after the injection of radiomethylmethionine (0.003 mmol/100 g weight of the rat).

methylaminoethanol, or dimethylaminoethanol in the normal diet had no appreciable effect on the extent of incorporation of the radiomethyl group of methionine into choline. Had the methyl groups of methylaminoethanol or dimethylaminoethanol, as constituents of choline, originated exclusively from methionine, the supply of the unlabeled methyl derivatives of ethanolamine in the diet would have "diluted out" the activity of the methyl groups of choline observed in the same rats which did not ingest the methyl derivatives of ethanolamine

TABLE VIII

Utilization of Methionine-CH_3-C^{14} for Choline Synthesis by Folic Acid-Deficient Rats which Received in the Diet a Continuous Supply of Ethanolamine, Methylaminoethanol, Dimethylaminoethanol, or Choline

Supplement fed with diet %	Standard specific activity of choline × 100
None	19.0
Ethanolamine, 0.34	15.4
Methylaminoethanol, 0.40	24.0
Dimethylaminoethanol, 0.45	37.1
Choline, 0.50	9.1
Citrovorum factor injected 2 hrs before isotope	34.8

Choline was isolated from the entire carcasses 20 hrs after the injection of radiomethylmethionine (0.003 mmol/100 g weight of the rat).

in the diet. The fact that the administration of choline together with radiomethylmethionine to normal rats produced such a "dilution" of the activity of tissue choline indicated to us that only the third group of choline originates from methionine.

The data in Table VIII show that the supplementation of the diet deficient in folic acid with ethanolamine or methylaminoethanol was without effect on the inhibited biosynthesis of choline from radiomethylmethionine. The presence of dimethylaminoethanol in the deficient diet, however, promptly increased the utilization of the radiomethyl group of methionine for choline formation. The administration of the *Citrovorum factor* to the deficient rats had a similar effect as the administration of dimethylaminoethanol.

RESULTS

We next ascertained if the results which we obtained on intact animals could be reproduced in liver slices. The data obtained on slices of livers of folic acid-deficient rats (Table IX) are in accord with those obtained on intact animals.

Our conclusions can be briefly summarized as follows: (1) The methyl groups of the dimethylaminoethanol moiety of choline originate *de novo* and that their neogenesis, probably from one-carbon units and their precursors, is mediated by a folic acid derivative, such as the *Citrovorum factor* or a compound which is derived from it *in vivo*, such as the 5,6,7,8-tetrahydrofolic acid. (2) The neosynthesized dimethylaminoethanol is the direct *in vivo* acceptor of the methyl of methionine, the transfer of which takes place via the trans-

TABLE IX

Synthesis of Choline from Methionine-CH_3-C^{14} by Slices of Livers of Folic Acid-Deficient Rats

Supplement to liver slices	Total activity recovered in choline *c.p.m.*
None	3,679
Ethanolamine	3,310
Methylaminoethanol	3,600
Dimethylaminoethanol	6,815
Ethanolamine plus *citrovorum factor*	7,277

20 μmol of methionine-CH_3-C^{14}. 20 μmol of the nonisotopic amines, 0.05 mg *Citrovorum factor* (Leucovorin, Lederle), 2 g liver slices, 25 ml Ringer phosphate buffer containing Mg^{++}, pH adjusted to 7.4. Incubated in air for 2 hrs at 37°. Carrier choline was added at the end of incubation, the slices were homogenized, and proteins were precipitated with TCA. The proteins were extracted with boiling ethanol, then with ether, the extracts were added to TCA filtrate, and choline was isolated from the combined extracts after saponification with alcoholic KOH.

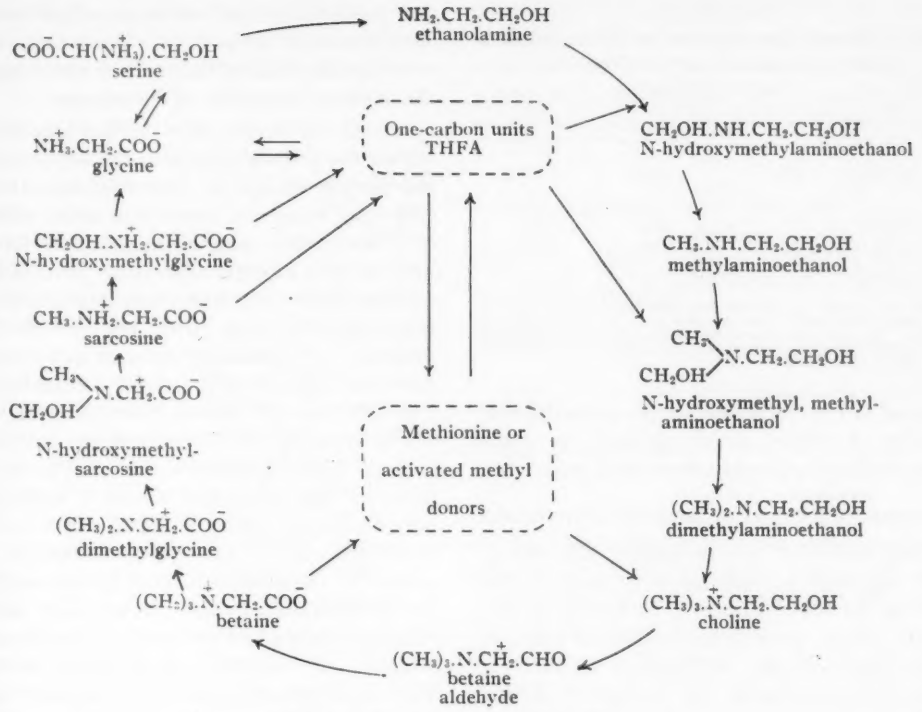


Fig. 2. A proposed schema for choline cycle.

methylation route. (3) The process of transfer of the methyl group of methionine to dimethylaminoethanol in the biosynthesis of choline does not involve folic acid or its derivatives.

Of interest in this connection are the studies of Nyc[30] on a *Neurospora* mutant which accumulates methylaminoethanol from ethanolamine. He found that the extent of incorporation of the carbon of radioformate into the methyl group of methylaminoethanol was seven times greater than that obtained when radiomethyl-methionine was used instead of the radioformate. These data strongly suggested that the methyl group of dimethylaminoethanol in this *Neurospora* mutant was derived via the *de novo* synthesis from formate rather than via the transmethylation from methionine. Nyc's[30] observations are consonant with our previously expressed views on the origin of the methyl groups of choline, although they lack proof that folic acid or its derivative was involved in the biosynthesis of methylaminoethanol from formate in this mutant. The available information on the role of folic acid derivatives in the processes of formate utilization and transfer leave little doubt, however, that in the *Neurospora* mutant, employed by Nyc,[30] the involvement of a folic acid derivative in the biosynthesis of methylaminoethanol from formate and ethanolamine will be possible to demonstrate and elucidate.

Earlier in this discussion we mentioned that the inability of the chick to utilize methionine for the synthesis of choline was apparently due to the limited biosynthesis of dimethylaminoethanol in this species.[12] Nutritional studies have shown that the addition of dimethylaminoethanol to chick diets, supplemented with methionine, satisfies the requirement for choline in the chick.[12] The data in Table X provide biochemical evidence for the participation of dietary dimethylaminoethanol in the biosynthesis of choline from methionine in the chick. As will be noted, the addition of dimethylamino-

TABLE X

Effect of Dimethylaminoethanol on Choline Synthesis from Methionine-CH_3-C^{14} in Chicks Fed Choline-Free Diets

Supplement to diet	Weight per chick in standard specific activity of choline	
	5 weeks g	× 100
None	234	12.4
Dimethylamino-ethanol	458	21.0

The diet, except for choline, was complete. 0.0067 mmol of radiomethylmethionine was injected per chick (3 birds per group); the birds were sacrificed 20 hrs after the injection of radiomethylmethionine, and choline was isolated from the pooled carcasses of each group.

ethanol to the chick diet, ample in methionine but free of choline, almost doubled the extent of biosynthesis of choline from methionine.

POSSIBLE MECHANISMS OF CHOLINE BIOSYNTHESIS

The data which we presented in this discussion suggested a need for a revision of the schema, shown in Figure 1, in which the hypothesis of the stepwise methylation of ethanolamine was outlined. In Figure 2 we have attempted to summarize our concept of choline biosynthesis and biodegradation. The schema shown in Figure 2 is necessarily incomplete, and it merely outlines the possible steps of various transformations, without precisely indicating the mechanisms and cofactors which are involved in each of the steps. Some facts, however, are known and they will be mentioned in the course of discussion of our scheme.

At the far upper right of the schema are shown the proposed steps in the neogenesis of the methyl groups of dimethylaminoethanol. The main feature of these steps is the transfer of a one-carbon component, probably formaldehyde or its biologic equivalent, to the ethanolamine moiety, the transfer being mediated by tetrahydrofolic acid, with the intermediate formation of N-hydroxymethyl-tetrahydrofolic acid from tetrahydrofolic and formaldehyde. The existence of N-hydroxymethyl-tetrahydrofolic acid has been demonstrated, and the recent evidence indicates that the carbon of formaldehyde in this compound forms a methylene bridge between the N-10 and N-5 of the tetrahydrofolic acid.[31] The next step could be visualized as the condensation of the tetrahydrofolic acid-formaldehyde derivative with aminoethanol, followed by cleavage of the tetrahydrofolic acid from the condensation product. The condensation with tetrahydrofolic acid-formaldehyde derivative with aminoethanol and the subsequent cleavage of the condensation product are mediated by specific enzymatic mechanisms. Whether N-hydroxymethyl-

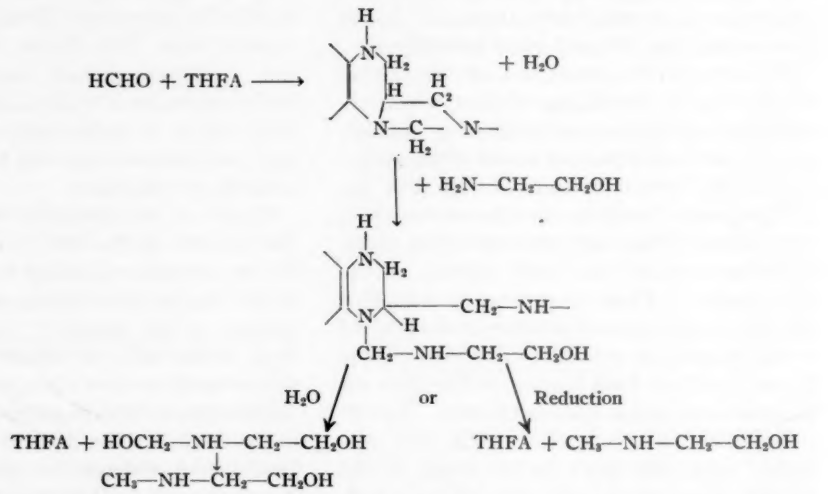


Fig. 3. A schema for the possible mechanism of biosynthesis of methylaminoethanol. (By analogy with the mechanism of serine synthesis proposed by Kisliuk, R. L.: *J. Biol. Chem.* 227:805, 1957.)

aminoethanol is formed as a result of the cleavage, as shown in the schema, or whether methylaminoethanol is formed directly during a reductive cleavage (possibly involving pyridine nucleotide in the reduction) is of course left open for future studies. The subsequent steps, leading to the formation of dimethylaminoethanol, could be similarly depicted via the mediation of another molecule of tetrahydrofolic acid-formaldehyde condensation product. Of course, another set of specific enzymatic mechanisms is probably involved in the steps leading to the production of dimethylaminoethanol from methylaminoethanol. These possible reactions are shown in Figure 3. Finally, dimethylaminoethanol accepts the methyl group of methionine via the process of transmethylation. It is quite likely that the actual donor of the methyl group to dimethylaminoethanol is not methionine but some "activated" form of methionine, possibly S-adenosyl-methionine sulfonium, which has been demonstrated to be the actual methyl donor in transmethylation reactions to creatine and N′-methylnicotinamide. Whether S-adenosyl-methionine is the actual methyl donor in the biosynthesis of choline from dimethylaminoethanol has not as yet been demonstrated.

As can be readily seen from the schema shown in Figure 2, the reactions shown for the biosynthesis of choline on the right side of the cycle are exactly the reverse of the steps for the degradation of choline via the intermediate formation of betaine by the action of choline dehydrogenase and betaine aldehyde oxidase shown on the left of the cycle. Betaine is the actual donor of the methyl group to methionine, via the mediation of betaine transmethylase, with the formation of dimethylglycine. The latter is degraded in a stepwise fashion by the successive action of dimethylglycine oxidase to sarcosine and formaldehyde, then by the action of sarcosine oxidase on sarcosine to glycine and formate-formaldehyde.[22] Whether or not intermediary N-hydroxymethyl derivatives of sarcosine and glycine are actually formed in the course of such biodegradation of dimethylglycine is not known, but their transient existence does not appear unlikely.

Of considerable interest are the recent studies by Sweeley and Horning[32] (Fig. 4) on the ferric ion-catalyzed reactions of dimethylglycine oxide in water over a pH range of 2 to 9. The products of the reaction were found to be a secondary amine and formaldehyde (reaction 2),

$$RCH_2N(CH_3)_2O \xrightarrow[H_2O]{Fe^{+++}} RCH_2N\begin{matrix}CH_3\\CH_2OH\end{matrix} \quad (1)$$

$$RCH_2N\begin{matrix}CH_3\\CH_2OH\end{matrix} \rightarrow RCH_2NHCH_3 + HCHO \quad (2)$$

$$RCH_2N(CH_3)_2O + HCHO \rightarrow RCH_2N(CH_3)_2 + HCOOH \quad (3)$$

(C. C. Sweeley and E. C. Horning, *J. Am. Chem. Soc.* 79: 2620, 1957.)

Fig. 4 Rearrangements and decarboxylation reactions of N,N-dimethylglycine oxide.

which were formed from a carbinolamine (reaction 1), and a tertiary amine and formic acid, which were formed from dimethylglycine oxide and formaldehyde (reaction 3).

Sweeley and Horning[32] suggest that the "successive steps of oxide formation, oxide rearrangement (reaction 1) and hydrolytic cleavage of a methylol intermediate (reaction 2) constitute a chemical model for demethylation, and methylation to a secondary or tertiary amine may be represented by the same sequence operating in a reverse direction." An enzyme-catalyzed amino acid oxide rearrangement corresponding to the transformations depicted in reactions 1, 2, and 3 has been observed with a mouse liver homogenate for L-N,N-dimethyltyrosine oxide and DL-N,N-dimethyltryptophan oxide,[33] although these oxides of the amino acid derivatives are not known to be cellular components. Sweeley and Horning[32] suggest the possibility that N-oxides are intermediates in biologic methylation-demethylation reactions. In the laboratory synthesis of dimethylaminoethanol from aminoethanol and formaldehyde[34] one could, therefore, postulate the intermediate formation of N-hydroxymethyl-methylaminoethanol; these possibilities are depicted in Figure 5. These chemical model reactions in the formation of N-methyl derivatives and in their oxidation serve as excellent analogies to the possible enzymatic re-

actions which are involved in the synthesis of dimethylaminoethanol and in the degradation of dimethylglycine with the mediation of a biological derivative of folic acid as the cofactor.

supported by the fact that the *de novo* synthesis of the methyl of methionine from any precursor of the one-carbon moiety was inhibited by the deficiency in folic acid.[35] A series of reactions, analogous to that shown in Figure 3, for the bi-

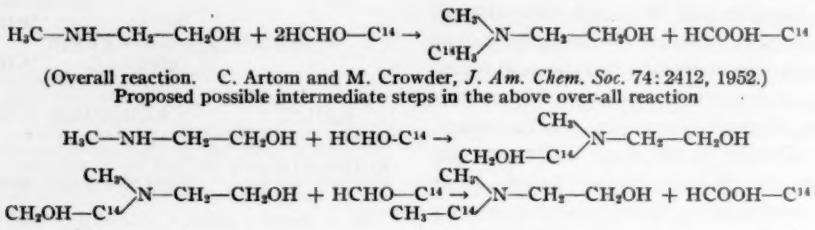


Fig. 5. A schema for the possible mechanism of the laboratory synthesis of dimethylaminoethanol.

It is within the realm of possibility that the biosynthesis of S-methyl group (methionine), or C-methyl group (thymine) from one-carbon compounds, such as formaldehyde, or from their precursors, such as serine, proceeds through the mediation of a biological derivative of folic acid, probably tetrahydrofolic acid, in a manner analogous to that outlined for the

osynthesis of the methyl group of methionine is shown in Figure 6.

The Role of Vitamin B_{12} in Neogenesis of Methyl Groups.

As to the role of vitamin B_{12} in the neogenesis of methyl groups, all that can be said at the present time is that, to our knowledge, it is not

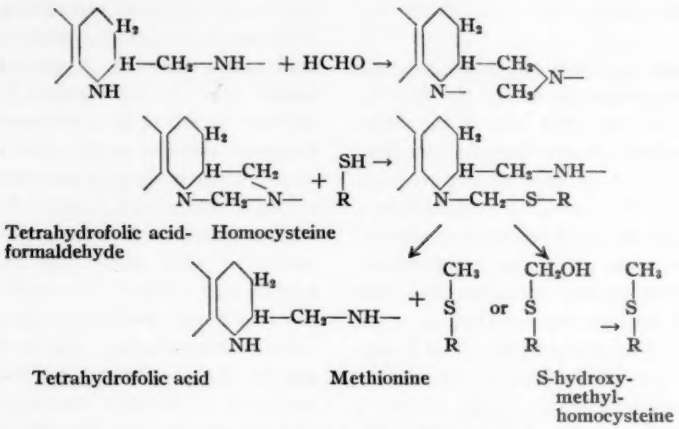


Fig. 6. Possible steps in methionine synthesis.

N-methyl groups of methylaminoethanol and of dimethylaminoethanol. Obviously, the apoenzyme components of the systems involved in these synthetic processes are different, although the nature of the cofactors in these systems could be related. Such a possibility is

known that vitamin B_{12} or its biologic equivalent constitutes a cofactor in any known enzymatic system. True, various proposals for the involvement of vitamin B_{12} as a cofactor in various enzymatic reactions, including the neosynthesis of methyl of methionine, have been

made by several authors. But no specific and unequivocal evidence to that effect has been published, as far as we know. This, of course, does not exclude vitamin B_{12} as a vital component of the living organism, nor does it imply that vitamin B_{12} is devoid of biologic activity. The published information on intact organisms indicated a reduction in the utilization of glycine-2-C^{14} for the incorporation into tissue choline and methionine in vitamin B_{12}-deficient rats,[36,37] mice and chicks,[38] swine,[39] turkey poults,[40] and a protozoa.[41] Other workers claimed that deficiency in vitamin B_{12} also reduces the extent of incorporation of radioformate and serine-3-C^{14} into tissue choline and methionine in rats[37] and in baby pigs and chicks.[42] Strangely enough, the extent of incorporation of radioformaldehyde carbon into tissue choline and methionine in vitamin B_{12}-deficient baby pigs and chicks was considerably greater than in normal animals.[42] The latter observation is particularly difficult to reconcile with the fact that the β-carbon of serine gives rise *in vivo* and *in vitro* to formaldehyde, and yet in the vitamin B_{12}-deficient baby pigs and chicks[42] the utilization of the β carbon of serine for choline and methionine was reduced and the utilization of formaldehyde was increased.

Schweigert and Wong[43] implicated vitamin B_{12} in the biosynthesis of nucleic acids, as was indicated by the decrease in the concentration of DNA and RNA per gram of liver of newborn rats of vitamin B_{12}-deficient dams, and by a decreased rate of DNA and RNA regeneration after partial hepatectomy in vitamin B_{12}-depleted growing rats. O'Dell and Bruemmer[44] also observed a lower extent of incorporation of P^{32} into the DNA and RNA in the vitamin B_{12}-depleted rats, and showed that this was related to a decrease in the rate of mitosis. Dubnoff[45] implicated vitamin B_{12} in the activation of the thiol groups of certain enzymes. Wagle and Johnson,[46] on the other hand, find that the deficiency in vitamin B_{12} has no effect on the nucleic acid biosynthesis, while it reduces the extent of incorporation of radioamino acids into liver proteins and into the microsome fraction of the liver and spleen.[47,48] These workers claim that vitamin B_{12} may serve as a cofactor in the biosynthesis of proteins, although no evidence could be claimed for the reduction in the incorporation of radiomethionine, glycine, or serine in the intact vitamin B_{12}-deficient rats[35] or chicks[18] into tissue proteins.

Totter[49] is of the opinion that "if the assumption is made that vitamin B_{12} in animal experiments functions solely to promote methyl group neogenesis (reduction of one carbon fraction to methyl) many diverse observations of its effects in nutrition can be reconciled." Such a reconciliation is not, however, evident from the brief résumé of the data which we just discussed. It would appear to us that experiments on intact animals cannot by their nature and design pinpoint the exact cofactor role for any vitamin, including vitamin B_{12}. At best, they can only indicate suggestive leads which must be followed up by studies on the more or less isolated systems. Clearly, the elucidation of the mechanisms of the bioreactions through which vitamin B_{12} deficiency affects the neogenesis of methyl groups,[34–40] biosynthesis of proteins and nucleic acids,[41,42,44,45] metabolism of carbohydrates and lipids,[51] and even glutathione,[50] the enhanced carcinogenicity of butter yellow in rats,[52] or the retardation of the carcinogenic properties of butter yellow,[53] cannot be profitably attempted at this time. Claims for the cofactor role of vitamin B_{12} in a bioreaction require considerably more evidence and of less equivocal nature than we now possess, and pending more exacting work to this end, we leave this problem to the future for the successful elucidation.

Choline Biosynthesis vs. Nutritional Requirement for Choline

The proposed scheme for biosynthesis of choline, as outlined in Figure 2, indicates that several moieties of choline, such as ethanolamine, the two methylated derivatives of ethanolamine, and the third methyl group which migrates to dimethylaminoethanol, originated *in vivo* via a diverse series of independent reactions. The over-all efficiency in choline formation, will, therefore, depend on the rate of the slowest reaction in the sequence. The nutritional requirement for choline in animals and other organisms is obviously determined by the

over-all efficiency of choline biosynthesis, and hence by the rate of the slowest reaction in the sequence that leads to the elaboration of choline. Furthermore, one should also take into account the rate of reactions which govern the catabolism of choline through the indicated oxidative paths.

Numerous nutritional observations on various species of living organisms in regard to choline requirement can be logically appraised in terms of the efficiency of the reactions which govern biosynthesis and biodegradation. For example, calves and weanling rats have a markedly low capacity to utilize methionine for choline synthesis, yet these animals do not have an absolute requirement for choline in the presence of methionine.[54,55] In these animals, however, the activity of choline dehydrogenase, and hence the capacity to oxidize choline to betaine, is also markedly reduced,[55,56] thus apparently enabling the animals to compensate for the relatively low capacity to form choline from methionine. In contrast, as we have mentioned earlier, the requirement for choline in the chick and the guinea pig, in the presence or absence of methionine, is determined by the low activity in these species of the enzymatic mechanisms which elaborate dimethylaminoethanol. Although in the guinea pig and in the chick the capacity to oxidize choline to betaine is also relatively low, this biologic conservation device for sparing choline is apparently insufficiently effective to eliminate completely the requirement for choline in the presence of dietary methionine.

Griffith and co-workers[57] have shown that the weanling rat is much more susceptible to hemorrhagic kidney lesions than the older animal, the male being the most sensitive in this respect. It would appear that the age as well as the sex of the animal could also be considered as contributory factors to the over-all efficiency of the reactions involved in choline biosynthesis. It seems desirable to correlate the efficiency of the various steps that are involved in choline biosynthesis with such factors as the species, age, sex, etc., with the over-all requirement for dietary choline. Such a study seems particularly pertinent in man, because the only biochemical fact about the biosynthesis of choline in man is that deuteriomethylmethionine, fed to man, yields liver choline (plasma phospholipids) which contains deuterium in the methyl groups.[58] What is the precise mechanism of choline biosynthesis in man? In the sequence of reactions leading to choline biosynthesis, which reaction constitutes the limiting step in man? The answers to these questions would be of help in the evaluation of choline requirement in man.

Of interest in this connection is the recent report on the remarkable efficacy of dimethylaminoethanol, as contrasted to choline, in schizophrenics.[59] The authors believe that the efficacy of dimethylaminoethanol, is due to a greater extent of synthesis of acetylcholine from dimethylaminoethanol, contrasted to that from choline, because of limited transport of choline across the membranal barriers and because of rapid oxidation of choline to betaine. If we assume, as we must, that dimethylaminoethanol serves as the intracellular precursor of choline in man, would it be justifiable to conclude *a priori* that dietary choline should be biologically equivalent to dimethylaminoethanol? In other words, is dietary choline a complete biologic substitute for the entire series of reactions that operate *in vivo* in the elaboration of choline? Or must we grant the possibility that the intracellularly elaborated choline is a more effective precursor of certain vital biologic components of the tissue than the dietary choline can be? If this possibility is granted, then the consideration of the reactions which lead to the elaboration of dimethylaminoethanol *in vivo*, as the direct precursor of choline and its derivatives, acquires additional importance. Perhaps these considerations should be kept in mind in situations in which the use of choline in various clinical disorders is recommended.

REFERENCES

1. Jukes, T. H.: *Ann. Rev. Biochem.* 16: 239, 1947.
2. Artom, C.: *Phosphorus Metabolism*, Vol. II (Ed. by W. D. McElroy and B. Glass). Johns Hopkins Press, Baltimore, 1952.
3. Stetten, D. W.: *J. Biol. Chem.* 140: 143, 1941; 144: 50, 1942.
4. Levine, M. and Tarver, H.: *J. Biol. Chem.* 184: 427, 1950.

5. GREENBERG, D. M. and HARRIS, S. C.: *Proc. Soc. Exper. Biol. & Med.* 75: 683, 1950.
6. STEKOL, J. A., WEISS, S., SMITH, P., and WEISS, K.: *J. Biol. Chem.* 201: 299, 1953.
7a. BEST, C. H., HERSHEY, J. M., and HUNTSMAN, M. E.: *Am. J. Physiol.* 101: 7, 1932.
7b. BEST, C. H. and HUNTSMAN, M. E.: *J. Physiol.* 75: 405, 1932.
8a. TUCKER, H. F. and ECKSTEIN, H. C.: *J. Biol. Chem.* 121: 479, 1937.
8b. CHANNON, H. J., MANIFOLD, M. C., and PLATT, A. P.: *Chem. and Ind.* 57: 600, 1938.
8c. SINGAL, S. A. and ECKSTEIN, H. C.: *Proc. Soc Exper. Biol. & Med.* 41: 512, 1939.
9a. DU VIGNEAUD, V., CHANDLER, J. P., MOYER, A. W., and KEPPEL, D. M.: *J. Biol. Chem.* 128: cviii, 1939.
9b. DU VIGNEAUD, V., CHANDLER, J. P., MOYER, A. W., and KEPPEL, D. M.: *J. Biol. Chem.* 131: 57, 1939.
10. SIMMONDS, S., COHN, M., CHANDLER, J. P., and DU VIGNEAUD, V.: *J. Biol. Chem.* 149: 519, 1943.
11. JUKES, T. H.: *J. Nutrition* 20: 445, 1940.
12a. JUKES, T. H. and OLESON, J. J.: *J. Biol. Chem.* 157: 419, 1945.
12b. JUKES, T. H., OLESON, J. J., and DORNBUSH, A. C.: *J. Nutrition* 30: 219, 1945.
13. REID, M. E.: *J. Nutrition* 56: 215, 1955.
14. HOROWITZ, N. H.: *J. Biol. Chem.* 162: 413, 1946.
15. DU VIGNEAUD, V.: *A Trail of Research.* Cornell Univ. Press, Ithaca, N. Y., 1952.
15a. WELCH, A. D. and NICHOL, C. A.: *Ann. Rev. Biochem.* 21: 663, 1952.
16a. COHEN, S. S. and WEED, L. L.: *J. Biol. Chem.* 209: 789, 1954.
16b. GREEN, M. and COHEN, S. S.: *J. Biol. Chem.* 225: 387, 1957.
17. WEYGAND, F., WACKER, A., TREBST, A., and SWOBODA, O. P.: *Ztschr. Naturforsch.* 12b: 184, 1957.
18. STEKOL, J. A., WEISS, S., ANDERSON, E. I., PENG, T. H., and WATJEN, A.: *J. Biol. Chem.* 226: 95, 1957.
19. NOLAND, G. L. and BAUMANN, C. A.: *Proc. Soc. Exper. Biol. & Med.* 70: 198, 1949.
20a. STEENSHOLT, G.: *Acta Physiol. Scandinav.* 10: 333, 1945.
20b. BARRENSCHEEN, H. K.: *Ztschr. f. physiol. Chem.* 284: 228, 1949.
21. VETCH, F. P. and ZWEIG, G.: *J. Am. Chem. Soc.* 74: 1921, 1952.
22. MACKENZIE, C. G.: *Amino Acid Metabolism* (Ed. by W. D. McElroy and B. Glass). Johns Hopkins Press, Baltimore, 1955.
23a. KELLER, E. B., RACHELE, J. R., and DU VIGNEAUD, V.: *J. Biol. Chem.* 177: 733, 1947.
23b. DU VIGNEAUD, V., RACHELE, J. R., and WHITE, A. M.: *J. Am. Chem. Soc.* 78: 5131, 1956.
24. ERICSON, L. E., HARPER, A. E., WILLIAMS, J. N., and ELVEHJEM, C. A.: *J. Biol. Chem.* 219: 59, 1956.
25. ERICSON, L. E., WILLIAMS, J. N., and ELVEHJEM, C. A.: *J. Biol. Chem.* 212: 537, 1955.
26. SLOANE, N. H., BOGGIANO, E. M., COULOMB, B., and HUTCHINGS, B. L.: *Fed. Proc.* 15: 357, 1956.
27. KELLEY, B., TOTTER, J. R., and DAY, P. L.: *J. Biol. Chem.* 187: 529, 1951.
28. STEKOL, J. A., WEISS, S., and ANDERSON, E. I.: *J. Am. Chem. Soc.* 77: 5192, 1955.
29. DU VIGNEAUD, V., CHANDLER, J. P., SIMMONDS, S., MOYER, A. W., and COHN, M.: *J. Biol. Chem.* 164: 603, 1946.
30. NYC, J. F.: *J. Biol. Chem.* 223: 811, 1956.
31a. BLAKLEY, R. L.: *Biochem. J.* 58: 448, 1954.
31b. KISLIUK, R. L.: *J. Biol. Chem.* 227: 805, 1957.
32. SWEELEY, C. C. and HORNING, E. C.: *J. Am. Chem. Soc.* 79: 2620, 1957.
33. FISH, M. S., SWEELEY, C. C., JOHNSON, N. M., LAWRENCE, E. P., and HORNING, E C.: *Biochem. & Biophys. Acta* 21: 196, 1956.
34a. CLARKE, H. T., GILLESPIE, H. B., and WEISSHAUS, S. E.: *J. Am. Chem. Soc.* 55: 4571, 1933.
34b. ARTOM, C. and CROWDER, M.: *J. Am. Chem. Soc.* 74: 2412, 1952.
35. STEKOL, J. A.: *Amino Acid Metabolism* (ed. by W. D. McElroy and B. Glass). Johns Hopkins Press, Baltimore, 1955.
36. STEKOL, J. A., WEISS, S., and WEISS, K. W.: *Arch. Biochem.* 36: 5, 1952.
37. ARNSTEIN, H. R. V. and NEUBERGER, A.: *Biochem. J.* 55: 259, 1953.
38. STEKOL, J. A., WEISS, S., HSU, B., and SMITH, P.: *Fed. Proc.* 11: 292, 1952.
39a. CHANG, I. and JOHNSON, B. C.: *Arch. Biochem.* 55: 151, 1955.
39b. JOHNSON, B. C., FIRTH, J., and MISTRY, S. P.: *Arch. Biochem.* 54: 467, 1955.
40. VOHRA, P., LANTZ, F. H., and KRATZER, F. H.: *J. Biol. Chem.* 221: 501, 1956.
41. JOHNSON, B. C., HOLDSWORTH, E. S., FORD, J. E., PORTER, J. W. G., and KON, S. K.: *Biochem. J.* 60: xxxix, 1955.
42. MISTRY, S. P., RAMA RAO, P. B., and JOHNSON, B. S.: Abstr. Am. Chem. Soc. Meeting, New York, Sept., 1957.
43. WONG, W. T. and SCHWEIGERT, B. S.: *J. Nutrition* 58: 231, 1956.
44. O'DELL, B. L. and BRUEMMER, J. H.: *J. Biol. Chem.* 227: 737, 1957.
45. DUBNOFF, J. and BARTRON, E.: *Arch. Biochem.* 61: 99, 1956; 62: 86, 1956.
46. WAGLE, S. R. and JOHNSON, B. C.: *Fed. Proc.* 16: 401, 1957.
47. WAGLE, S. R. and JOHNSON, B. C.: *Arch. Biochem.* (in press). Quoted by WAGLE, S. R. *et al.* in *J. Am. Chem. Soc.* 79: 4249, 1957.
48. WAGLE, S. R., MEHTA, R., and JOHNSON, B. C.: *J. Am. Chem. Soc.* 79: 4249, 1957.
49. TOTTER, J. R.: *Ann. Rev. Biochem.* 26: 181, 1957.
50. LING, C. T. and CHOW, B. F.: *J. Biol. Chem.* 198: 439, 1952.

51. LING, C. T. and CHOW, B. F.: *J. Biol. Chem.* 206: 797, 1954.
52. DAY, P. L., PAYNE, L. D., and DINNING, J. S.: *Proc. Soc. Exper. Biol. & Med.* 74: 854, 1950.
52a. MILLER, E. C., PLESCIA, A. M., MILLER, A. J., and HEIDELBERGER, C.: *J. Biol. Chem.* 196: 863, 1952.
53. BENNETT, M. A., RAMSEY, J., and DONNELLY, A. J.: *Internat. Rev. Vit. Res.* 26: 417, 1956.
54. STEKOL, J. A., PENG, T. H., ANDERSON, E. I., and WEISS, S.: Abstr. Am. Chem. Soc. Meeting Cincinnati, March, 1955.
55. HOPPER, J. H. and JOHNSON, B. C.: *Proc. Soc. Exper. Biol. & Med.* 91: 497, 1956.
56. MONDY, N. I., STRENGTH, D. R., GRAY, L. F., and DANIEL, L. J.: *Proc. Soc. Exper. Biol. & Med.* 87: 129, 1954.
57. GRIFFITH, W. H.: *J. Nutrition* 19: 437, 1940.
58. SIMMONDS, S. and DU VIGNEAUD, V.: *J. Biol. Chem.* 146: 685, 1942.
59. PFEIFFER, C. C., JENNEY, E. H., GALLAGHER, W., SMITH, R. P., BEVAN, W., JR., KILLIAM, K. F., KILLIAM, E. K., and BLACKMORE, W.: *Science* 126: 610, 1957.

DISCUSSION

Dr. R. Abrams (Montefiore Hospital, Pittsburgh, Pa.): One specific question has occurred to me. This concerns the transmethylation experiments of du Vigneaud *et al.* using a methyl donor doubly labeled with C^{14} and deuterium. Dr. Stekol cited this work where the ratio of deuterium to C^{14} was the same in the methyl group of choline formed as in the precursor. It was implied that this transfer, whether it involved one methyl or two or three, should maintain a constant ratio of deuterium to C^{14} in the methyl groups. However, the data you have presented indicated that at least two of these methyls are oxidized to one carbon unit and are then transferred to choline by reactions involving tetrahydrofolic acid. These data imply that, in terms of an average choline methyl group, you should find a decreased ratio of deuterium to carbon^{14}. So there does seem to be a discrepancy, which perhaps Dr. Stekol might clarify.

Second, would you care to comment on the fact that you do seem to think there is some sort of role of B_{12} in methyl group neogenesis starting from the alpha carbon of glycine or the beta carbon of serine? We know from the work of Buchanan and the work of Rabinowitz on purine degradation, where purified enzymes have been used, that there does not seem to be a role for B_{12} in the reactions involving one carbon unit and tetrahydrofolic acid. The question is, at what step in methyl group biogenesis could B_{12} come in?

In that connection, what is your feeling about the nonspecific role of B_{12} which has been proposed by Dubnoff? With, I think, a B_{12}-less mutant he finds that very many SH enzymes seem to be activated by B_{12}, which presumably functions in the liberation of SH's necessary for activity.

I chose to read the part of the paper by Pfeiffer and co-workers that did *not* concern schizophrenics. If I may quote from this paper on the effects of dimethylaminoethanol on normal (nonschizophrenic) subjects: "Oral doses of 10 to 20 mg/day produce a mild and pleasant degree of central nervous system stimulation characterized by lessened daytime fatigue, by sounder sleep, and fewer hours of sleep are required. In addition, dimethylaminoethanol produces relief from periodic headache, bowel distress, and chronic fatigue."

Dr. J. F. Herndon (Smith, Kline & French Laboratories, Philadelphia, Pa.): I was very much interested in the points about the origin of choline. The question came to mind as to just how one distinguishes between dimethylaminoethanol and choline when one gives dimethylaminoethanol? It seems to me that it would be very difficult to separate the two compounds. Can you do it with the reinecke salts? How would you do this?

Dr. Stekol: It can be done, you know. Dr. Artom can tell you about that. There is a method for it.

Dr. Artom: Yes, this can be done, but not with reinecke salt, because of a partial coprecipitation of dimethylethanolamine from its mixtures with choline. In the method elaborated by Miss Crowder and myself (*Fed. Proc.* 8: 180, 1940), after destruction of primary and secondary amines with HNO_2, dimethylethanolamine is separated by steam distillation. Sizable amounts were determined in both the lipids and the aqueous extracts of the tissues of rats, shortly after a large dose of the compound had been given. However, we have not been able to detect dimethylethanolamine in the tissues of normal rats. It may be that this compound is present only in very small amounts, which could well fit in with its postulated role as a metabolically active intermediate. Still, it would be very nice if we could demonstrate its presence in the tissues without question.

Dr. Harper (University of Wisconsin, Madison, Wis.): I was glad to see Dr. Stekol's outline because it explains some of the discrepancies in results we obtained in an attempt to compare the lipotropic activity of a number of substances that I shall discuss. I would, also, like to ask whether Dr. Stekol has an explanation for the fact that four different groups of people, one of whom does not actually believe that B_{12} is involved in transmethylation reactions, did find lowered activity of the transmethylation system in vitamin B_{12}-deficient rats. If that evidence is accepted, is it possible that the enzyme activity could be decreased somewhat in B_{12}-deficient animals without being decreased sufficiently to affect the total amount of isotope that would be transferred from betaine to methionine?

Dr. Stekol (closing remarks): I shall answer the last question first. One of the four groups mentioned by Dr. Harper happens to be ourselves, who also found

with rat liver homogenate of B_{12}-deficient rats that there is a decrease in the utilization of homocysteine for the synthesis of methionine from betaine. We don't know why. It can be reproduced. However, when betaine homocysteine transmethylase was isolated there was not a trace of vitamin B_{12} in it. This enzyme could not be resolved into a cofactor and apoenzyme. It is electrophoretically pure. It does not mean much, but still it is as clean as you can make it. There is neither folic acid nor folic acid activity in it; and vitamin B_{12} or cobalt is zero. Administration of radioactive B_{12} to rats and the re-isolation of betaine transmethylase also resulted in complete absence of B_{12} in betaine transmethylase.

For that reason we believe that experiments with the homogenate, the earliest ones by Oginsky and those by ourselves and then by Erickson and your group, are inconclusive. Really, if you look at the data a little closer, they do not prove anything. You yourselves found no B_{12} in your cofactor that you claim to have isolated. The difference between the extent of methionine formation in the deficient animal liver homogenate and the normal ones was so small that I don't think you can attribute it to the decrease in the activity of betaine transmethylase.

Besides, the measurements of methionine in your case, I think, were done colorimetrically by Sullivan's procedure which, by the way, has a limit in accuracy. Later, we and others used bacteriologic procedures for methionine determination and found no difference whatever in methionine formation from betaine and homocysteine between normal livers and those deficient in B_{12}. That is where the matter stands.

I doubt very much if vitamin B_{12} is involved in transmethylation reactions because even nutritional studies prove that animals can grow and fatty infiltrations are alleviated on vitamin B_{12}-free diets, if methionine is administered. Choline is being made from methionine in vitamin B_{12}-deficient animals, and that they are deficient in B_{12} is indicated by the fact that they die.

With regard to Dr. Artom's statement about the inability to detect dimethylethanolamine, only recently has homocysteine been detected in the tissues. The fact that some intermediates are very difficult to detect is no proof of their absence. Besides, dimethylethanolamine is very rapidly methylated in animal tissues, and the extent of formation of phospholipid choline from dimethylethanolamine amounts to about 80 per cent of that formed by choline itself. So, the incorporation of dimethylethanolamine into choline of phospholipid is rapid, and for that reason you would not be able to find free dimethylethanolamine hanging around, unless methods for its detection are considerably improved.

Regarding the efficacy of dimethylethanolamine in schizophrenics mentioned by Dr. Abrams, I think we brought this up to emphasize the need for a study of mechanism of choline formation in the human being.

Dr. Abrams also mentioned the isotopic studies of du Vigneaud. If we very carefully examine du Vigneaud's data, it will be found that everything he says is exactly right if you follow his procedure. We have had almost a lifetime of experience with du Vigneaud, and it is very difficult to find him wrong. The description of the technics used in the doubly-labeled methionine experiments has to be very carefully noted. They are all short-term experiments. When doubly-intramolecularly-labeled methionine was administered, the animals were killed very shortly, afterwards (three days) the livers removed and for some reason discarded. Choline was isolated from the carcasses.

Your point is that if the methyl group of methionine is oxidized to formate and formaldehyde and then reincorporated back into the methyl and the chain of choline, how is it that when you isolate the tissue choline you still have the same ratio of carbon^{14} to deuterium in the methyl groups? If you do it under short-term conditions, the time necessary for oxidation and reincorporation is not available. If, however, you do a long-term study with intramolecularly labeled methionine, you will have a gradual alteration in the ratio and appearance of the carbon^{14} in the ethanolamine moiety of choline. I believe the time allowed was three days or something of that sort. As you could see, the C^{14} units from radiomethylmethionine go through a large cycle. There are nearly twelve steps involved before the carbon of methionine as formate will reincorporate itself back into methionine. If sufficient time is not allowed, you will not have alteration in the ratio. If you do, you will.

The design and intent of du Vigneaud's experiments were to prove the existence of transmethylation, and that he did.

Dr. Abrams: Are there any figures on the turnover time of choline?

Dr. Stekol: Yes, I think Dr. Zilversmit will have that.

Dr. Zilversmit: No, I don't have them.

Dr. Stekol: I can quote Stetten, three studies by Boxer, and what we reported about three or four years ago. The half-life of choline in the liver of rats fed choline-containing diets is about six hours. The half-life of carcass choline is about twelve days. So I think what is to be done to check that point is self-evident. There is no question regarding the methyl group carbon of methionine being oxidized to formate, that the formate or formaldehyde incorporates into serine, or that β-carbon of serine appears in ethanolamine or the ethanolamine moiety of choline. Du Vigneaud reports that the choline that he isolated had no isotopic carbon in the ethanolamine moiety. Incidentally, the oxidation of the methyl group of methionine to formate was reported from du Vigneaud's laboratory by McKenzie. So it is not a contradiction, and yet it is. It is a question of how much methionine you feed to rats and how long you let rats percolate with the isotopic methionine in them.

The Biosynthesis of Phospholipids

EUGENE P. KENNEDY, PH.D.*

A CURRENT theory of the mechanism of the lipotropic action of choline and inositol suggests that these substances exert such an effect because they are important constituents of phospholipids. It is not known, however, precisely how the metabolism of phospholipids may be related to lipotropic activity. The interesting and important experiments of Dr. Zilversmit, discussed in this Symposium may shed some light on this problem. In this paper I will discuss some current work on the enzymatic synthesis of phospholipids, with the hope that some clue as to the mechanism of the lipotropic action of choline may emerge. Three main topics are to be considered: the enzymatic synthesis of lecithin; the enzymatic synthesis of sphingomyelin; and some preliminary experiments on the level of CDP-choline† in the livers of rats on a choline-deficient diet.

THE ENZYMATIC SYNTHESIS OF LECITHIN

The enzymatic processes by which lecithin is built up from simple precursors in the living cell has now been worked out in considerable detail. Some of these reactions are summarized in Figure 1. Since this subject has been reviewed recently elsewhere[1,2] the present discussion will be limited to features particularly pertinent to the lipotropic action of choline and to the choline-containing phospholipids. It should be mentioned however that an essentially similar scheme of reactions leads to the formation of phosphatidyl ethanolamine.

PC-Cytidyl Transferase Reaction: It has now been firmly established that a coenzyme form of choline, cytidine diphosphate choline (CDP-choline), is an essential intermediate in the bio-synthesis of lecithin. The structure of CDP-choline, which is that of a doubly substituted pyrophosphate, is shown in Figure 2. The synthesis of CDP-choline and related compounds in labeled form by purely chemical procedures has been described.[3] The enzymatic synthesis of CDP-choline from CTP + P-choline [reaction (e) of Fig. 1] is catalyzed by PC-cytidyl transferase, an enzyme widely distributed throughout nature. A detailed study of this enzyme has recently been carried out by Borkenhagen and Kennedy.[4]

The PC-cytidyl transferase reaction may be viewed as an "activation" of phosphorylcholine by incorporation into the energy-rich pyrophosphate structure of CDP-choline, and is one of the many reactions for which metabolic energy is required to carry out the biosynthesis of phospholipid.

PC-Glyceride Transferase Reaction: CDP-choline reacts with a D-1,2-diglyceride [reaction (f) Fig. 1] to form lecithin and CMP (cytidine-5′-phosphate). The enzyme catalyzing this reaction has been named PC-glyceride transferase and its properties have been described by Smith, Weiss, and Kennedy.[5] The enzyme requires magnesium or manganese ion and is severely inhibited by very low concentrations of calcium ion.

The CMP which is formed in reaction (f) may be rephosphorylated to CTP at the expense of ATP. The CTP which is thus regenerated may combine with another mole of phosphorylcholine, participating in a catalytic

---

From the Department of Biochemistry, University of Chicago.

* Professor of Biochemistry, University of Chicago.

Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc., New York, New York.

This work has been supported by grants from the Nutrition Foundation, the Life Insurance Medical Research Fund, and the National Institute for Neurological Diseases and Blindness U.S.P.H.S. (B-1199).

† CDP-choline = cytidine diphosphate choline; CoA = coenzyme A; CTP = cytidine triphosphate; ATP = adenosine triphosphate; CMP = cytidine-5′-phosphate.

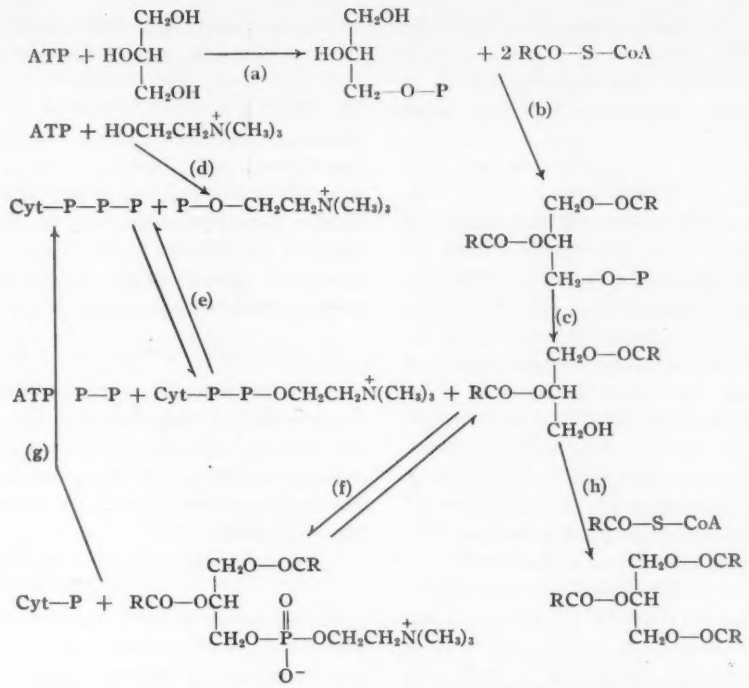


Fig. 1. Pathways for the enzymatic synthesis of phospholipids and triglycerides.

cycle, at every turn of which one mole of lecithin is formed from phosphorylcholine + diglyceride. During the course of this cycle, phosphorylcholine forms a part of the structure of the coenzyme itself, in a fashion somewhat analogous to the function of the uridine coenzymes, e.g., uridine diphosphate glucose.

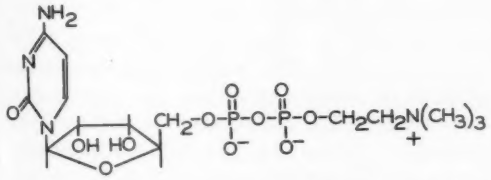


Fig. 2. The structure of cytidine diphosphate choline.

The PC-glyceride transferase is specific for CDP-choline; analogues in which adenosine, uridine, or guanosine replace the cytidine portion of the molecule have been prepared synthetically and are completely inactive. The enzyme is also highly specific for D-1,2-diglycerides; 1,3-diglycerides are only slightly active. The D-1,2-diglyceride must possess at least one unsaturated fatty acid, preferably in the alpha-prime position, and must be emulsified in a suitable nonionic detergent such as "Tween-20" to penetrate to the enzyme surface. The requirement for an unsaturated fatty acid may be related to the greater ease of emulsification of unsaturated glycerides.

A recent generous gift by Drs. E. Baer and D. Buchnea of samples of pure synthetic D-

TABLE I

The Optical Specificity of the PC-Glyceride Transferase

Diglyceride added	Lecithin synthesized $m\mu M$
1. L-1,2-diolein	13
2. D-1,2-diolein	126

The assay for lecithin synthesis was carried out as described by Weiss, Smith, and Kennedy.[5]

and L-1,2-diolein has made it possible to test the optical specificity of the enzyme, with the results shown in Table I. The D-1,2-diglyc-

eride is seen to be much more active than the L-enantiomorph.

The PC-glyceride transferase reaction is freely reversible, which may be of some physiologic importance.

Interrelationships of Phospholipid and Triglyceride Synthesis: The scheme shown in Figure 1 indicates that L-α-phosphatidic acids and D-1,2-diglycerides may be intermediates of hitherto unsuspected importance in the biosynthesis of glycerophosphatides. [For references, see review.][2] This conclusion is supported by the discovery[6] that D-1,2-diglycerides may react with long-chain thioesters of coenzyme A to form triglycerides, and thus may be regarded as precursors of neutral fat as well as of phospholipides. Several laboratories have sought an alternative pathway for the formation of triglyceride, i.e., a direct reaction between free glycerol and long-chain thioesters of coenzyme A. To date no such reaction has been found, and the only known route for the incorporation of free glycerol into triglycerides is by way of L-α-glycerophosphate, phosphatidic acid, and D-1,2-diglyceride.

Is CDP-Choline a Lipotropically Active Form of Choline? One of the great advances in achieving an understanding of nutrition on a biochemical level has been the realization that water-soluble vitamins are of special importance in the diet because they are building blocks for coenzymes. It has further been learned that in general the vitamin does not function as a coenzyme as such, but is transformed in the body to a catalytically active form, often a nucleotide.

Although choline is not a vitamin in the strict sense of the word, its importance as a special dietary factor is well recognized. Since we now know that choline may be converted in the body to a catalytically active nucleotide form, the hypothesis that CDP-choline is a lipotropically active form of choline deserves careful consideration. The close interrelationship between triglyceride and phospholipid metabolism shown in Figure 1 makes this hypothesis all the more attractive, since the profound alteration in the triglyceride metabolism of liver, resulting in fatty liver, is one of the most dramatic consequences of a diet deficient in choline and other lipotropic agents. To consider but one obviously oversimplified interpretation, any condition which disturbs the balance between reactions (f) and (h) by blocking reactions (d), (e), or (f) (Fig. 1) might lead to a "spilling over" of 1,2-diglyceride into neutral fat rather than phospholipid. Reactions (d), (e), or (f) might be blocked or reduced either by an insufficient supply of choline to the cell or by metabolic poisons such as carbon tetrachloride.

THE ENZYMATIC SYNTHESIS OF SPHINGOMYELIN

The experimental results which have just been described suggested that the biosynthesis of another choline-containing phospholipid, sphingomyelin, might take place by a reaction essentially similar to the PC-glyceride transferase reaction:

(1) Cyt-P-P-choline + N-acylsphingosine $\rightleftharpoons$ Sphingomyelin + CMP

It has recently been reported[7] that an enzyme from chicken liver catalyzes an extensive net synthesis of sphingomyelin according to reaction (1). The name phosphorylcholine-ceramide transferase has been proposed for this enzyme.

Phosphorylcholine-ceramide transferase is highly specific both for CDP-choline and for N-acylsphingosine (ceramide). Ceramides with fatty acids of short or intermediate chain length in amide linkage are much more active than the naturally occurring long chain compounds, presumably because the short chain compounds penetrate more readily to the enzyme surface. Much more surprising is the discovery[7] that the N-acylsphingosine must possess the *threo* rather than the *erythro* configuration in the sphingosine portion of the molecule. It has been generally accepted that naturally occurring sphingolipids have the *erythro* configuration. The physiologic significance of this puzzling observation merits further study.

The participation of CDP-choline in the biosynthesis of sphingomyelin makes it clear that the functions of this nucleotide form of choline are not limited to the realm of glycerophosphatides and strengthens the view that mechanisms controlling the synthesis and utili-

zation of CDP-choline may be of central importance in lipid metabolism generally.

MEASUREMENTS OF CDP-CHOLINE CONCENTRATION

It is now known that CDP-choline is widely distributed in nature[1] being found in the liver of various species including the rat and the hen, in brain and other tissues of the rat, and in yeast.

As a preliminary attempt to determine whether CDP-choline is a lipotropically active form of choline, it seemed of interest to determine the concentration of CDP-choline in the livers of rats under various conditions. Efforts to work out an enzymatic assay for CDP-choline have so far been unsuccessful, and it has been necessary to use an isotope dilution method. This procedure, although sensitive and accurate, is laborious and time-consuming and has therefore limited the number of experiments which can be done.

In one experiment, 18 male rats, about five weeks old, were divided into two groups of nine each. One group was fed *ad libitum* on a commercially available choline-deficient diet (Nutritional Biochemicals Co.). The control group was fed the same diet, supplemented with choline. After eight weeks, both groups of animals were killed. The livers of three animals were pooled for CDP-choline assay, resulting in three assays for each group. The results are shown in Table II. There was no

TABLE II

CDP-Choline Content of Rat Liver in Choline-Deficient and Control Animals

$\mu M/100$ g

	I	II	III
Control	6.2	7.1	6.2
Deficient	5.9	6.9	5.9

significant difference between control and deficient animals.

These results, although very fragmentary, seem to suggest that the level of CDP-choline in liver may be relatively constant, even on a choline-deficient diet. However, other experiments, unfortunately incomplete, seem to indicate that the CDP-choline content of the livers of choline-deficient animals first falls, and then returns to normal, so that a period of eight weeks may be too long to detect such changes.

It is clear that much more extensive and better planned experiments will be needed to explore these points.

REFERENCES

1. KENNEDY, E. P.: The metabolism of lipides. *Ann. Rev. Biochem.* 26: 119, 1957.
2. KENNEDY, E. P.: The biosynthesis of phospholipides. *Fed. Proc.* (in press).
3. KENNEDY, E. P.: The synthesis of cytidine diphosphate choline, cytidine diphosphate ethanolamine, and related compounds. *J. Biol. Chem.* 222: 185, 1956.
4. BORKENHAGEN, L. F. and KENNEDY, E. P.: The enzymatic synthesis of cytidine diphosphate choline. *J. Biol. Chem.* 227: 951, 1957.
5. WEISS, S. B., SMITH, S. W., and KENNEDY, E. P.: The enzymatic formation of lecithin from cytidine diphosphate choline and 1,2-diglyceride. *J. Biol. Chem.* (in press).
6. WEISS, S. B. and KENNEDY, E. P.: The enzymatic synthesis of triglycerides. *J. Am. Chem. Soc.* 78: 3550, 1956.
7. SRIBNEY, M. and KENNEDY, E. P.: The enzymatic synthesis of sphingomyelin. *J. Am. Chem. Soc.* 79: 5325, 1957.

DISCUSSION

Dr. Wilgram: Dr. Kennedy, I am wondering whether I am right in assuming that you think that the most important findings in this field in the last year or two are that 1-α-glycerophosphate is a very important precursor of D-1,2-diglycero-phosphatidic acid and that this compound, after fission of the phosphate group then reacts with cytidine diphosphate choline to form lecithin.

From the biochemical viewpoint there is nothing that I can add to your presentation at all, but as a physiologist there is some amazement about the fate of the phosphate group. After the formation of phosphatidic acid, phosphate is split off. I am wondering why Nature should take such a complicated and roundabout course, first, in putting phosphate on 1-α-glycerophosphate to yield the phosphatidic acid, and then removing it by a phosphatase, only to put another phosphate from CDP-choline back in the same position. From a physiologic viewpoint it seems to me that Nature, if it takes this course, is somewhat wasteful and I am wondering what your personal reactions are as to this complicated phenomena.

The second point I would like to ask you again arises from physiologic and not biochemical considerations. There are certain aspects of the problem which have been touched upon but not dealt with extensively by Dr. Kennedy. They are however, vital to the understanding of what goes on in the living

body. How does this biochemical finding of the mode of enzymatic synthesis of lecithin in the test tube lead to a better understanding of the mode of action of choline, in the intact organism, which is the main topic of the symposium today? You have mentioned several possible mechanisms by which neutral fat and phospholipids may be interrelated to each other in their metabolism. I wonder whether you would care to indicate which possibilities, if any, you think might account for the lipotropic action of choline.

Dr. Kennedy: With regard to the first point that Dr. Wilgram made, we are not responsible for how Nature chooses to make these compounds. Yet I think it would be unfortunate if the impression got around that Nature really would do well to study biochemistry rather than vice versa. I think there is some rhyme or reason to this merry-go-round in which the glycerol is phosphorylated, converted to phosphatidic acid and then dephosphorylated, because when the glycerol is phosphorylated it is phosphorylated stereospecifically, giving only L-α-glycerophosphate. Then when the phosphatidic acid is formed, it has the L configuration. It is a derivative of L-α-glycerophosphate but by a quirk in the arbitrary nomenclature of these compounds, the compound which is formed by splitting the phosphate from an L-phosphatidic acid is a D-1,2-diglyceride. That is simply because you change the end of the molecule you regard as the precursor of the aldehyde of D-glyceraldehyde. It gives specifically then the form of 1,2-diglyceride which is needed to react with cytidine diphosphate choline to given an L-lecithin. As far as we can tell, all of the glycerophosphatides in nature are of the L series.

This is exactly what Baer does when he makes lecithins synthetically. He takes the D-acetone-glycerol molecule and makes the benzyl ether to maintain stereospecificity. He puts the fatty acids on and then removes the benzyl ether and phosphorylates, using chemical methods.

Actually, there is quite a strict analogy between the chemical synthesis of lecithin and the biochemical synthesis of lecithin. In each case a protective group is put on in the alpha position while the fatty acids are introduced in order to preserve stereochemical purity, and then the protector is removed.

I realize this does not answer completely your question, which has real force to it. We see again and again that enzymatic reactions occur which seem at first to be wasteful, for example, splitting out of pyrophosphate rather than orthophosphate in certain reactions. I think there is a deeper physiologic significance, as you point out, and I cannot cope with it at that level at the moment.

As far as the second question is concerned, which of these interrelationships between triglyceride and phospholipid one might regard as a fruitful point of departure for investigating the lipotropic activity of choline, all I can say is that my instinct tells me it will not be as simple as blocking cytidine diphosphate choline utilization and switching the diglyceride over to triglyceride. It can't be that simple in view of the immense literature on the lipotropic activity of choline, almost unrivaled for its complexity. On the other hand, I think it would be extraordinary, really, if we found a cofactor form of choline which had no relationship at all to the lipotropic activity of choline because there are so many examples—nicotinamide, for example, in which the dietary nutrient is incorporated into a nucleotide or cofactor form and then exerts its biological activity. I have no evidence at all to cite that CDP-choline has lipotropic activity, but I still think it would be a good bet in the long run.

Dr. W. Cornatzer (University of North Dakota, Grand Forks, N. D.): The data on Dr. Kennedy's last slide where he found the cytidine diphosphate choline content in fatty livers identical to the control animals are similar to other data where no change in phospholipid turnover was observed in production of dietary cirrhosis (*Ann. New York Acad. Sc.* 57: 919, 1954) or liver necrosis (*J. Lab. & Clin. Med.* 31: 478, 1946). The composition of liver lipids can be altered by dietary intake of proteins. There is usually a slight decrease in the level of total phospholipids in the liver of animals maintained on a low-protein diet. The decrease is especially marked in the lecithin fraction with a consequent lower ratio of choline-containing to total phospholipids. The uptake of P^{32} or phospholipid turnover, however, is the same in the fatty livers as in the controls.

Dr. H. Segal (University of Pittsburgh, Pittsburgh, Pa.): I would like to comment on Dr. Kennedy's remark regarding the reversibility of the transfer of phosphoryl choline from CDP-choline to glyceride. I think this is perhaps not so surprising. An analogous situation is the reversibility of polynucleotide phosphorylase. It apparently means that we have to include phospho diesters as high energy phosphate compounds.

Dr. Kennedy (closing remarks): I would like to agree that the glyceride transferase reaction and polynucleotide synthesis are analogous. However, at least to my mind it was unexpected that the phospholipids present in considerable amount in the cell could represent an unsuspected reserve of "high energy" compounds. Whether this has any physiologic meaning at all I do not know.

Role of Choline in the Hepatic Oxidation of Fat ?

CAMILLO ARTOM, M.D.*

THE HYPOTHESIS that phospholipids, especially liver phospholipids, are related to the metabolism of fatty acids was suggested a long time ago by Loew,[1] who stated that lecithin was "a machine for burning fats." Later Leathes[2] speculated that, as a first stage of their catabolism, fatty acids are incorporated into liver phospholipids and in this form undergo an initial process of desaturation. The findings that after administration of fat, increases in the amounts[3] and in the rate of the synthesis of phospholipids[4] occurred in certain tissues, but not in others, was interpreted as indicating the existence of two classes of phospholipids, functionally distinct.[4,5] The liver, plasma, intestinal mucosa, and perhaps also the kidney, were postulated to contain a high proportion of "lipometabolic phospholipids," specifically involved in fat metabolism, whereas in the other tissues, phospholipids represented chiefly structural materials, "cytoplasmic phospholipids."

Since the nitrogenous constituent of lecithins, choline is lipotropic[6] it seemed logical to identify the lipometabolic phospholipids with the lecithins, or possibly, with some specific lecithin. This interpretation seemed even more likely, when it was found that choline stimulated the turnover of liver phospholipids,[7,8] and that this stimulation was enhanced, when fat was also administered.[9] Other substances, such as cystine,[10] or ethanolamine,[11,12] which do not show lipotropic activity, were found to enhance the incorporation of P^{32} into liver phospholipids. But, unlike these substances, choline stimulated only,[13] or chiefly,[12] the turnover of lecithins.

Since phospholipids have hydrophylic groups, and lecithins are the major components of plasma phospholipids,[14] lecithins have also been considered as a suitable form for the transport of fatty acids in plasma from liver to peripheral tissues. Accordingly, it was reasoned that in choline-deficient animals the impairment in the synthesis of lecithins would result in an accumulation of fat in the liver, either because of a decreased oxidation of fatty acids in the tissue itself, or because of an inadequate mobilization in the form of plasma lecithins. The results of several investigations, in which deuterium was used as a tracer, seemed to be more in line with the latter interpretation. In choline-deficient rats maintained on heavy water, the labeled fatty acids accumulating in the liver are chiefly the result of a synthesis in the liver, rather than of a transport from the depots to the liver.[15] In mice first fed heavy water, and then shifted to a deuterium-free, low-protein diet, no differences could be detected in the rates at which the labeled fatty acids disappeared from the liver, or from the depots, whether or not the diets had been supplemented with choline.[16] In a subsequent study, in which the synthesis of fatty acids from D_2O was investigated, the livers of choline-deficient rats contained greater amounts of newly synthesized fatty acids, whereas the depots contained the same amounts as, or lesser amounts than, those found in the animals receiving choline.[17] These findings have been recently confirmed and extended by Bernhard *et al.*[70] These and other investiga-

From the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina.

Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of the National Vitamin Foundation, Inc., New York.

* Professor and Head of the Department of Biochemistry, Bowman Gray School of Medicine.

This presentation is largely based on investigations carried out under a research contract (At-(40-1)-1638) between the U. S. Atomic Energy Commission and the Bowman Gray School of Medicine.

tors used C^{14}-acetate as a means of labeling the fatty acids of animals on diets deficient in choline. In short range experiments, Bernhard *et al.*[76] did not find any significant difference in the amounts of newly synthesized fatty acids present in the livers or the depots of rats on deficient diets, supplemented or not supplemented with choline. The results of experiments of longer duration, reported by Guggenheim and Olson,[77] and by Bernhard *et al.*[70] are in substantial agreement with those of similar experiments which have been carried out in our laboratory during the past several years. In these experiments the specific activities of the fatty acids synthesized in the livers and depots of choline-deficient and choline-supplemented rats and mice were the same. Obviously the investigations with D_2O or C^{14} acetate failed to give any indication that choline affects either the synthesis or the catabolism of fatty acids. Accordingly such investigations were interpreted as following the view that choline enhanced the transfer of newly synthesized fatty acids from the liver to the depots. If this is true, the mechanism still requires elucidation.

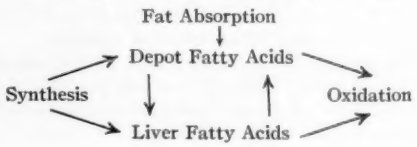
Numerous findings have made increasingly difficult the acceptance of the idea that phospholipids, themselves, are the major transport form for fatty acids. Hevesy and Hahn[18] found that when labeled serum phospholipids containing P^{32} made in one animal were injected into a second animal, most of the isotopic phospholipid was taken up by the liver. In similar experiments it was shown that if the liver is removed, or excluded from the circulation, labeled phospholipids disappear from the plasma at an extremely slow rate.[19] Moreover, in rats injected with inorganic P^{32}, choline administration increases phospholipid turnover in liver, but not in the plasma, the higher specific radioactivity of the plasma lipid P being merely the reflection of the high rate of phospholipid synthesis in the liver.[20] These results clearly suggested that there is a continuous exchange of phospholipids between liver and plasma, but not between plasma and other tissues. In reviewing these findings several years ago I concluded that it seemed more likely that the lipotropic effect of choline was due to an increased catabolism of fatty acids in the liver, but that much more direct evidence was needed before such a possibility could be accepted.[21]

I. *IN VITRO* OXIDATION OF FATTY ACIDS IN THE LIVERS OF PROTEIN DEFICIENT RATS

Herein is presented a schema* adapted from one drawn by Stetten and Salcedo[17] with modifications to incorporate the growing evidence that oxidation and synthesis of fatty acids occur extensively in both depots and liver. In view of the multiplicity of the processes which may affect the levels of fatty acids in the liver as well as in the depots, it is obvious that the interpretation of the results obtained on intact animals is complex and subject to much uncertainty. Accordingly, it was felt that perhaps a clear cut demonstration of a possible role of choline in the oxidation of fatty acids could be obtained more easily in experiments on isolated tissue preparations. As pointed out by Weinhouse *et al.*[22] in such experiments the use of fatty acids, labeled with C^{14}, seemed to be especially advantageous. Because of the sensitivity and specificity of radioactive measurements, changes in the rate of fatty acid oxidation are demonstrable, even if the total consumption of oxygen remains the same. Moreover, such changes can be easily distinguished from stimulations or inhibitions of the overall metabolism by other substances added *in vitro*. Last, since only minute amounts of substrates with high specific activity need to be used, one can avoid more or less completely the inhibition of oxidative processes which is caused by the *in vitro* addition of fatty acids, especially long-chain fatty acids.[22,23]

In our experiments,[24] emulsions of stearate, or palmitate, labeled in the carboxyl carbon, were added to preparations from the livers of rats, previously maintained on various diets. After three hours, the radioactivity of the respir-

* Sources and Fates of Liver and Depot Fatty Acids.



atory CO_2, and acetoacetate, was measured. Under these conditions, the livers of rats on a five per cent casein diet produced less $C^{14}O_2$ and less labeled acetoacetate than the livers of rats on a stock diet. In most cases, supplementation of the low-protein diet with choline restored the ability of the tissue to oxidize the added fatty acid at a high rate. Even more marked and consistent were the effects of large doses of choline injected shortly before the animals were killed. These results are exemplified by the data of Table I.*

TABLE I

$C^{14}O_2$ Production from Stearate-1-C^{14} by Liver Homogenates of Rats on Various Diets

	Values per mg N of liver			
	Diet			
	No. 26* + GAA† 1.0%		No. 26 + Choline 0.4%	Stock
	Rat A	Rat B‡	Rat C	Rat D
Respiratory $C^{14}O_2$: c/m	310	1335	603	770
Neutral fat: mg	15.5	3.0	2.3	0.3

* Diet 26: low-fat diet, containing 5% casein.

† Guanido-acetic acid.

‡ Injected with choline chloride (150 μM/100 g rat) two hours before death.

Differences in the production of $C^{14}O_2$ by the livers of rats receiving, or not receiving, choline were studied in tissue slices, homogenates, and particles separated from these homogenates (Table II and III). It seems, therefore, unlikely that such differences could be ascribed to differences in the permeability of the cell membranes for the substrate. On the other hand, since larger amounts of fats were generally present in the livers of choline-deficient rats, one cannot dismiss the possibility that the lower amounts of $C^{14}O_2$ produced by these livers were merely the result of a greater dilution of the isotopic substrate with a consequent lowering of the specific activity at the site of its oxidation. However, there was no inverse relationship between the fat contents of various livers and the amounts of $C^{14}O_2$ produced by these livers *in vitro*. Moreover, in the washed particles, from which practically all excess fat had been removed by the washings, the differences in the production of $C^{14}O_2$ were in the same direction and as large as in the unfractionated homogenates (Table III).

In some experiments we have also studied the distribution of C^{14} among the various lipid

* In this and many of the following tables the data have been referred to 1 mg of tissue N. However, identical conclusions could be drawn, if the values were expressed for the whole liver of a 100 g rat.

TABLE II

$C^{14}O_2$ and C^{14}-Acetoacetate Production from Stearate-1-C^{14} by Liver Slices and Homogenates

	Values per mg N of liver	
	Rat A (no dietary supplement)*	Rat B (choline in diet and injected)†
Slices (Exp. No. 10)		
Respiratory $C^{14}O_2$: c/min	27	199
C^{14}-acetoacetate: c/min	12	63
Neutral fat: mg	13.8	1.5
Homogenate (Exp. No. 27)		
Respiratory $C^{14}O_2$: c/min	150	517
C^{14}-acetoacetate: c/min	20	169
Neutral fat: mg	5.4	1.1

* Diet 26: Low-fat diet containing 5% casein.

† Choline chloride added to the diet (0.4%) and injected (250 μM/100 g rat in two doses, 0.75 and 1.5 hours before death).

TABLE III

$C^{14}O_2$ Production from Stearate-1-C^{14} by Liver Homogenates and Particles

	Values per mg N of liver	
	Rat A (no dietary supplement)*	Rat B (choline in diet and injected)†
Whole Homogenate		
Respiratory $C^{14}O_2$: c/min‡	145	655
Neutral fat: mg	13.6	0.8
Mitochondria		
Respiratory $C^{14}O_2$: c/min‡	125	1025
Neutral fat: mg	1.7	0.5

* Diet 26: 5% casein.

† Choline chloride added to the diet (0.4%) and injected. (250 μM/100 g rat in two doses, 2 hr and 1 hr before death).

‡ 3 hours' incubation in air.

fractions, separated by chromatography, first on silicic acid and subsequently on Amberlite IRA-400. As shown in Table IV, during the incubation notable amounts of the isotopic fatty acid are incorporated into the phospholipid and in the neutral fat fractions. This process appears to be only partially enzymatic, since a rather extensive incorporation of palmitate into these two fractions (especially in neutral fat) occurred even in the control flasks containing boiled tissue. Similar observations have been made previously by others.[25] At any rate, it is apparent that, in spite of the differences in the fat content, approximately the same proportion of the isotopic palmitate was esterified as glyceride, or as phospholipid, in the two livers. Moreover, the comparison of the amounts of C^{14} in the CO_2 produced and in the free fatty acid remaining at the end of the experiment clearly suggests that the oxidation has occurred at the expenses of the free fatty acid only. The results of other experiments, in which the C^{14} content of acetoacetate was also determined, led to the same conclusion. There is therefore little doubt, at least in our mind, that the isotopic contents of the CO_2 and acetoacetate produced during the incubation represent a valid measurement of the oxidation of the fatty acid, added to the liver preparations.

TABLE IV

Distribution of C^{14} after Incubation of Liver Homogenates with Palmitate-1-C^{14}*

	Rat A (Diet 26)†	Rat B (Diet 26 + choline)†	Control flasks‡
C^{14} in§:			
Respir. CO_2	9.5	29.0	0.5
Free fatty acids	45.7	9.3	84.7
Neutral fat	21.7	18.6	11.3
Phospholipids	19.6	20.7	3.6
Neutral fat:• mg	147.0	19.4	
Phospholipids:• mg	19.5	18.5	

* 3 hr incubation in air.

† 5% casein, low-fat diet, unsupplemented (Rat A), or supplemented with choline, 0.4% (Rat B).

‡ Liver homogenate (Rat A) maintained at 100° for 10 min prior to the incubation.

§ Values in per cent of the total counts recovered in the control flask.

• Values per one gram of liver.

Such a process is depressed in the liver of rats on low-protein diets and it is enhanced by the administration of choline *in vivo*. In commenting on these results, I stated[24] that while the findings offered a reasonable explanation of the lipotropic action of choline, the possibility was not ruled out that other mechanisms might also be involved, as suggested by the earlier experiments on intact animals.

On the other hand, the *in vitro* addition of choline was uniformly ineffective, nor was any stimulation of fatty acid oxidation observed, when simple derivatives of choline such as phosphorylcholine, betaine aldehyde, or betaine* were added *in vitro*. We therefore postulated that the factor, or factors, responsible for the enhancement of fatty oxidation observed after choline administration was not free choline, but some substance formed from choline *in vivo*, and not under the conditions of our experiments *in vitro*. Since, as mentioned before, the differences between the livers of rats receiving, or not receiving, choline were demonstrable also in washed particles and in mitochondria, one should think of compounds held firmly in the granular structures, such as the choline-containing phospholipids.

Our observations may be compared with a number of other results obtained with nonisotopic technics. Several years ago Abdon and Börglin[26] noted that oxidative processes were decreased in the skeletal muscle of rats on protein deficient diets, and that choline, administered *in vivo*, but not *in vitro*, prevented such a decrease. According to Potter and Klug[27] and Lévy,[28] the ability to oxidize added octanoate is markedly depressed in homogenates of the livers of rats fed low-protein, high-fat diets. Administration of choline to these animals stimulated the oxidation of octanoate, whereas *in vitro* addition of choline, or phosphorylcholine, was not effective.[29]

More recently, under conditions similar to those of our experiments, Fritz[30] also observed

* Supplementation of a low-casein diet with betaine raised the *in vitro* oxidation of labeled fatty acids, although the increases were less marked than in the livers of rats on choline supplemented diets. Terminal injections of betaine were ineffective (C. Artom, unpublished results).

a decreased production of $C^{14}O_2$ from isotopic palmitate, added to homogenates of the livers of rats previously maintained on a low casein diet. *In vivo* administration of choline to these rats markedly enhanced the process. Only a slight degree of stimulation was observed, when rats on a stock diet were injected with choline.

In a further investigation we have also studied the oxidation of fatty acids in tissues other than the liver.[31] The results indicate that administration of choline to protein-deficient rats enhances fatty acid oxidation not only in the liver, but also in the kidney and heart (Table V). These findings are of interest, in view of

TABLE V

Effects of Choline Administration on Oxidation of Palmitate-1-C^{14} in Tissue Homogenates

Rats on Diet 26, containing 5% casein. Average relative values per mg N of tissue*

	Liver	Kidney	Heart	Brain	Testis
Choline-Cl injected†	217‡	120‡	136‡	106	99
In diet§ and injected†	210‡	182‡	198‡	135	94

* $C^{14}O_2$ produced by the tissues of rats not receiving choline = 100. Incubated in air for 3 hours.

† 0.250 μM/100 g rat in two doses, 2 hours and 1 hour before death.

‡ Significantly different from 100 ($P < 0.05$).

§ Choline Cl, 0.4%.

the occurrence of necrotic lesions in the kidney[32] and in the heart and large vessels[33,34] of rats on choline-deficient diets. Wilgram *et al.*[35] have pointed out that these changes are preceded by an accumulation of fat which can be prevented by supplementation of the diet with choline.

In other types of fatty liver, for which choline deficiency is not directly responsible, there is no definite evidence of an impaired oxidation of fatty acids. Indeed, conflicting reports have been published on the O_2 uptake, or on the production of ketone bodies by slices of livers damaged with phosphorus or carbon tetrachloride.[36–38] More recently, we have studied the oxidation of C^{14}-stearate added to homogenates of the livers of rats which had received a series of injections of CCl_4 in oil. The data of the individual experiments were rather irregular. However, from the average values, it would appear that, while the livers of the rats injected with CCl_4 were quite fatty, the rate of fatty acid oxidation in these livers was not significantly different from that observed in the livers of control rats which had received the oil without CCl_4.

II. LIVER LECITHINS AND FATTY ACID OXIDATION

As mentioned before, it seemed likely that choline, administered *in vivo*, enhanced the oxidation of fatty acids *in vitro* by promoting the synthesis of phospholipids, especially lecithins. Indeed, increases in the oxygen uptake have been noted, when lecithins, or products of the partial hydrolysis of lecithins, were added to mitochondria, isolated from the livers of rats, or guinea pigs.[39,40] In the latter type of preparations, phosphorylcholine stimulated the over-all oxygen uptake, but not the oxidation of C^{14}-palmitate. Oxidation of this substrate, however, was enhanced by the addition of lysolecithin, or glycerol-phosphoryl-choline in the presence of coenzyme A.

In many of our experiments on the livers of rats maintained on low-protein diets we have compared the effects of choline administration on the *in vitro* oxidation of fatty acids and on the amounts of choline-containing phospholip-

TABLE VI

Effects of Choline Administration on the Choline Containing Phospholipids and on Fatty Acid Oxidation in Liver Homogenates

Rats on low-protein diets. Average values per mg N of liver

No. of determinations*	Choline administered	Choline-containing phospholipids		$C^{14}O_2$ from fatty acids†	
		mg	Change %	*c/m*	Change %
7 (7)	None	0.33		104	
8 (8)	In diet	0.42	+27‡	216	+107‡
22 (38)	None	0.35		110	
30 (49)	Injected terminally	0.45	+29‡	230	+109‡
11 (28)	None	0.37		107	
10 (25)	In diet and injected	0.56	+51‡	358	+234‡

* Number of rats in parentheses.

† Homogenates incubated for 3 hours in air with stearate-1-C^{14} or palmitate-1-C^{14}. Values as per cent of the counts in the substrate × 10^2.

‡ Significantly different from O ($P < 0.05$)

ids.[41] The results of these experiments have been grouped according to the mode of administration of choline, and the average values of each group are recorded in Table VI. Variations of the individual data around the averages were rather large, possibly because in each group of experiments a number of factors, such as the fat content of the diets, the time on the diet, and the age of the animals, varied considerably. Previous investigations have indicated that the effectiveness of dietary choline for maintaining high levels of lecithins in the liver is dependent on several such factors.[42,43] It is apparent, however, that in all three groups of experiments, choline administration has caused increases in both the lecithin levels and the *in vitro* oxidation of fatty acids, and that such increases are statistically significant.

A similar rough parallelism between these two effects of choline was exhibited in experiments in which protein-deficient rats were injected with one large dose of choline and killed at various time intervals between 0 and 180 minutes after the injection (Table VII).

TABLE VII

Choline-Containing Phospholipids and Fatty Acid Oxidation in Liver Homogenates at Various Times After Injection of Choline

Rats on low-protein diets. Average values per mg N liver (4 experiments)				
Hours after injection choline*	Choline-containing phospholipids		$C^{14}O_2$ from fatty acids†	
	mg	Change %	*c/m*	Change %
0	0.273	—	93	—
0.5	0.333	+22	191	+106
1.0	0.402	+47	159	+ 71
3.0	0.494	+81	209	+120

* 150 μM choline-Cl per 100 g rat in one injection.

† Liver homogenates incubated for 3 hours in air with palmitate-1-C^{14}. Values as per cent of the counts in the substrate × 10^2.

In other investigations,[41] we have compared the changes in the fat content, in the lecithin level and in the rate of fatty oxidation, resulting from the administration of substances which might be expected to interfere, directly or indirectly, with the synthesis of choline or with its incorporation into liver lecithins. In one series of experiments, rats were maintained on low-protein diets, unsupplemented, or supplemented with either triethylcholine, or choline. From the average values recorded in Table VIII, it would appear that the effects of the unnatural analogue resemble those of choline in causing a decrease in the fat content of the liver and increases in both the lecithin level and the rate of fatty acid oxidation. However, the data of the individual experiments were rather irregular, and, especially in those experiments in which the animals had been on the experimental diet for longer periods, triethylcholine was only slightly, or not at all, effective. It might be noted in this respect, that while a lipotropic effect of triethylcholine has been reported by some authors,[44] in other conditions such an effect was not apparent.[45]

TABLE VIII

Lipids and Fatty Acid Oxidation in Liver Homogenates of Rats Receiving Triethylcholine, Choline, or no Supplements

Rats on low-protein diets. Average values per mg N of livers			
	Substances added to the diet*		
	Triethyl-choline	Choline	None
Neutral fat: mg	2.48	2.25	4.04
Phospholipids			
Choline-containing: mg	0.46	0.47	0.33
Total mg	1.00	0.95	0.82
C^{14} from fatty acids†			
Respiratory CO_2: *c/m*	144	230	128
Acetoacetate: *c/m*	134	148	70
Total *c/m*	278	378	198
No. of determinations	12	12	11

* Triethylcholine 0.65 g, or choline-Cl 0.5 g, added to 100 g of diet, after a depletion period.

† Homogenates incubated for 3 hours in air with stearate-1-C^{14}. Values as per cent of the counts of the substrate × 10^2.

We next compared the effects of supplementing the low-protein diets with diethanolamine, choline, or guanidoacetate, the last substance being supposed to make more severe the deficiency of choline[46] (Table IX). Previous investigations in this laboratory indicated that diethanolamine, added to low-protein diets, caused a decrease in the rate of the synthesis of liver phospholipids, as measured by the in-

TABLE IX

Lipids and Fatty Acid Oxidation in Liver Homogenates of Rats Receiving Diethanolamine, Choline, or Guanidoacetate

Rats on low-protein diets. Average values per mg N of liver	Substances added to the diet*		
	Diethanolamine	Choline	Guanidoacetate
Neutral fat: mg	0.94	1.21	5.16
Phospholipids			
Choline-containing: mg	0.17	0.42	0.36
Total: mg	1.14	1.03	1.06
C^{14} from fatty acids†			
Respiratory CO_2: c/min	163	177	80
Acetoacetate: c/min	99	118	45
Total: c/min	262	295	125
Number of determinations	13	9	12

* Diethanolamine 0.5 g, choline-Cl 0.5 g, or guanidoacetic acid 1.5 g, added to 100 g of diet, either immediately, or after a depletion period.

† Liver homogenates incubated for 3 hours in air with stearate-1-C^{14}. Values as per cent of the counts of the substrate × 10^2.

corporation of P^{32}, and marked changes in the phospholipid composition of the tissue.[47] These changes have been found again in the present experiments.[41] In the livers of rats which had received diethanolamine for several days, the choline-containing fraction is decreased, often much more markedly than in the livers of the controls on the choline-deficient diets. At the same time there occurred a considerable increase in the noncholine containing fraction. These changes are exactly opposite to those induced by choline supplementation. On the other hand, with diethanolamine as well as with choline, the *in vitro* oxidation of fatty acids is enhanced and the fat content remains low. These findings, in conjunction with others obtained in our laboratory more recently, suggest that diethanolamine interferes with the incorporation of choline into lecithins without actually impairing the synthesis of choline itself.

In another series of experiments, female rats on a stock diet, have been injected with large doses of DL-ethionine under the conditions described by Jensen *et al.*[48] Other animals received both ethionine and methionine, or ethionine and choline, or a saline solution only. All rats were killed 24 hours after the first injection. The average data (Table X) show that, in the livers of the rats injected with ethionine, there was an increase in liver fat and a very considerable decrease in the oxidation of fatty acids *in vitro*. Both changes were prevented by methionine injected together with ethionine. Choline, injected with ethionine, was ineffective and even might have increased the effects of ethionine, especially the accumulation of fat. On the other hand, the levels of the total and of the choline-containing phospholipids in the livers of rats receiving ethionine, or ethionine and choline, remained as high as in those of the control rats which had been injected with NaCl, or with the mixture of methionine and ethionine.[41] It might be pointed out that in dogs receiving ethionine over a rather prolonged period, Fein-

TABLE X

Lipids and Fatty Acid Oxidation in Liver Homogenates of Rats Receiving or not Receiving DL-Ethionine

Female rats on a stock diet, then fasted for 24 hours. Average values per mg N of liver	Substances injected*			
	Ethionine	Ethionine + Choline	Ethionine + methionine	NaCl
Neutral fat: mg	2.68	3.12	0.59	0.70
Phospholipids				
Choline-containing: mg	0.56	0.49	0.52	0.41
Total: mg	1.23	1.20	1.11	0.99
C^{14} from fatty acids†				
Respiratory CO_2: c/min	36	9	213	219
Acetoacetate: c/min	27	3	116	172
Total: c/min	63	12	329	391
Number of determinations	11	5	10	5

* Total amounts injected: DL-ethionine 1.3 mM, choline-HCl 0.65m mM, DL-methionine 1.3 mM, NaCl 0.9% 12 ml, per 100 g rat (4 injections).

† Liver homogenates incubated for 3 hours in air with stearate-1-C^{14}, or palmitate-1-C^{14}. Values as per cent of the counts of the substrate × 10^2.

berg *et al.*[49] and Furman *et al.*[50] observed a progressive decrease in all lipid and lipoprotein fractions of blood plasma. On the basis of their observations, the authors ascribe the fatty livers of dogs receiving ethionine to a decreased mobilization of fats, possibly because of an impaired synthesis of plasma lipoproteins in liver. A similar defect in the synthesis of enzyme proteins or lipoproteins could perhaps explain the strong inhibition of fatty acid oxidation which we have observed in our "acute" experiments. Such inhibition could be at least one factor for the production of fatty livers in the rats injected with ethionine. At any rate, it does not appear that choline deficiency was responsible, since the effects of ethionine were not prevented by the administration of choline. On the other hand, in a few experiments in which we have studied the incorporation of P^{32}, we could not detect any marked inhibition of the synthesis of lecithins in the liver of rats injected with ethionine.

In Table XI, the results of our various groups of experiments[41] are summarized and compared also with previous data on the effects of choline, triethylcholine, and diethanolamine on the synthesis of liver lecithins.[12,13,47,51] It appears that, with all four substances tested, changes in the rate of fatty acid oxidation *in vitro* are accompanied by opposite changes in the degree of fatty infiltration. However, whereas choline and triethylcholine cause increases in the synthesis and in the level of liver lecithins and also enhance fatty acid oxidation, such a parallelism is not maintained after administration of ethionine or diethanolamine. The results obtained with the latter compound indicate that a low-fat content and a high rate of fatty oxidation *in vitro* can be attained, even when the rate of the synthesis and the level of lecithins in the liver are markedly depressed.

TABLE XI

A Comparison of the Effects of the Administration of Choline, Triethylcholine, Diethanolamine, or Ethionine

Increases over (+), or decreases from (−) the controls				
			Choline-containing phospholipids	
Substances administered	Fatty infiltr.	Fatty acid oxid.	Level	Rate of synthesis*
Choline†	−	+	+	+
Triethylcholine†	−	+	+	+
Diethanolamine†	−	+	−	−
Ethionine‡	+	−	±	±

* Experiments with P^{32}.
† Controls on choline-deficient diets.
‡ Controls on stock diet.

The possibility remains that the enhancement of fatty acid oxidation by choline administration *in vivo* is due to the increased formation either of a choline derivative other than a phospholipid, or of one type of lecithin which exerts some specific role and may be a minor component of the whole choline-containing fraction of liver phospholipids.

Several reports indicate that phospholipids are essential constituents of important enzymes, such as the ATPases of skeletal muscle,[52] or liver nuclei.[53] The lipoprotein nature of a DPN-cytochrome *c* reductase isolated from rat muscle has been described recently.[54] Moreover, integrated enzyme systems, such as succinoxidase preparations or mitochondria where many oxidative processes, including the beta-oxidation of fatty acids and the reactions of the Krebs cycle, do occur, are rich in phospholipids. When these preparations are treated with phospholipases of various types, a number of the enzymes present are inactivated, and the inactivation parallels the destruction of phospholipids. In the recent experiments of Lévy and Legrand,[55] the ability of liver homogenates to oxidize octanoate was suppressed by lecithinase D of *Clostridium Welchii*, whereas the endogenous respiration was only partially inhibited. The effects of the lecithinase were, at least to a large extent, prevented by the addition of serum-containing specific antibodies against the enzyme. Likewise, treatment of mitochondria or of other polyenzymatic preparations by detergents, by lipid solvents, or by substances which may interact with phospholipids, also inactivate many of the enzymes present in such preparations.*

It is of course possible that phospholipids are essential for the orderly occurrence of complex

* For a bibliography of these topics the reader is referred to several reviews.[56–59]

processes involving a number of individual reactions (such as fatty acid oxidation) not necessarily because phospholipids are integral components of individual enzymes, but because they contribute in maintaining the spatial configuration of these enzymes in the granular structures of the cells. Evidence for the latter interpretation may be found in the observation that a partial enzymatic splitting of phospholipids inactivates succinic oxidase, whereas the individual components (succinic dehydrogenase and cytochrome oxidase) are still quite active.

From another viewpoint, it may well be that the existence of common pathways for the synthesis of phospholipids and neutral fats, which has been pointed out by Dr. Kennedy, will some day offer a basis for interpreting the apparent relationship between fatty acid metabolism and phospholipid turnover in the liver.

Whatever will turn out to be the final explanation, it seems unlikely that it will include the hypothesis of an actual incorporation of fatty acids into phospholipids, as a preliminary stage of fatty acid catabolism. Indeed, there is no definite place for such a stage in the present scheme of fatty acid catabolism, which has been established much more firmly and clarified to a very considerable degree during the last few years.

III. DIETARY FACTORS OTHER THAN CHOLINE INVOLVED IN FATTY ACID OXIDATION

The low-casein diets used in our previous experiments on the *in vitro* oxidation of isotopic fatty acids contained little methionine and were practically free of cystine. Accordingly, we also studied the effects of supplementing these or similarly deficient diets with sulfur-containing amino acids.[60] In Table XII, we have compared the average results of experiments, carried out simultaneously on the livers of rats which had been maintained for the same periods of time on various deficient diets, unsupplemented, or supplemented with cystine, choline, or methionine. It is apparent that any one of these supplements added to the experimental diets enhanced the oxidation of fatty acids *in vitro*, the increases being of about the same order of magnitude for each of the three substances tested.

TABLE XII

Effects of Dietary Cystine, Choline, or Methionine on Fatty Acid Oxidation in Liver Homogenates

Rats on diets low in cystine and methionine. Substrate: stearate-1-C^{14}, or palmitate-1-C^{14}. Average relative values per mg N

	Dietary supplements*		
	Cystine	Choline	Methionine
Respiratory $C^{14}O_2$†	277‡	254	274‡
Number of experiments	15	11	8

* L-cystine 1, choline chloride 0.4, DL-methionine 1%.

† Livers of rats on the unsupplemented diets = 100.

‡ Significantly different from 100.

In other experiments, the effect of tocopherol administration was tested.[60] Indeed it is known that a massive liver necrosis occurs frequently with diets low in S-containing amino acids, and that complete protection can be obtained by supplementing these diets with cystine, or with tocopherol, or with the so-called factor 3 (which is apparently an organic compound of selenium).[61] Table XIII summarizes the data of experiments, in which tocopherol had been omitted from the vitamin mixture, and the animals had been maintained on various low-protein diets, without or with the addition of tocopherol, or cystine, or both tocopherol and cystine. The results indicate that tocopherol alone is quite effective in maintaining a high rate of oxidation of fatty acids added *in vitro*.

TABLE XIII

Comparison of Effects of Dietary Cystine and/or Tocopherol on Fatty Acid Oxidation in Liver Homogenates

Rats on diets low in cystine and methionine. Substrate: stearate-1-C^{14} or palmitate-1-C^{14}. Average relative values per mg N

	Dietary supplements*		
	Cystine	Tocopherol	Cystine + tocopherol
Respiratory $C^{14}O_2$†	194‡	361‡	203‡
Number of experiments	11	10	7

* Cystine 1.5, tocopherol 0.025%.

† Livers of the rats on the unsupplemented diets = 100.

‡ Significantly different from 100 ($P < 0.05$).

Supplementation of the deficient diets with either cystine, or tocopherol, or both, also enhanced the synthesis of fatty acids in liver slices incubated with acetate-1-C^{14}. On the other hand, choline injected terminally in the animals, stimulated the oxidation, but not the synthesis of fatty acids *in vitro*. The effects of choline injected into rats previously maintained on deficient diets, unsupplemented, or supplemented with cystine, are summarized in Table XIV.

TABLE XIV

Effects of Choline Injected Terminally on Fatty Acid Oxidation, or Synthesis, in Liver Homogenates, or Slices

Rats on diet 26, containing 5% casein. Average relative values per mg N liver

Dietary supplement*	Choline injected†	Homogenates: CO_2 from palmitate-C^{14}‡	Slices: fatty acids from acetate-C^{14}‡
GAA	−	100	100
	+	373§ (7)	89 (9)
Cystine	−	241§ (11)	311§ (9)
	+	402§ (6)	166 (9)

* Guanidoacetic acid (GAA) 1.5, cystine 1.5%.

† 250 μM of choline Cl/100 g rat, in two doses, 2 hours and 1 hour before death.

‡ No. of experiments in parentheses.

§ Significantly different from 100 ($P < 0.05$).

Because of these apparent differences in the effects of choline and cystine on the oxidation and on the synthesis of fatty acids, respectively, we suggested that choline and cystine act on fatty acid metabolism through different mechanisms and at different biochemical sites. In this respect, the possibility was mentioned that the effects of cystine, and perhaps of tocopherol also, were due to the maintenance of high levels of coenzyme A in the tissue. Indeed in the livers of rats on cystine-deficient rats, Olson and his associates have found decreased amounts of CoA[62] and a lower rate of incorporation of cysteine-S^{35} into CoA.[63]

Likewise, changes in the amounts or in the intracellular distribution of other factors, such as glutathione,[64] cytochrome C,[65] and especially pyridine nucleotides,[62,65–68] have been described in various conditions which lead to fatty or necrotic livers (including choline deficiency).

In a number of unpublished experiments, we have added several of these factors to preparations of the livers of rats which had been maintained on deficient diets, unsupplemented or supplemented with either choline or cystine. The factors studied included: ATP, DPN, CoA, cysteine, CoA and cysteine, cytidine triphosphate and cytidine-choline diphosphate.* In unfractionated homogenates, ATP generally stimulated fatty acid oxidation, whereas with most other factors, inhibitions rather than stimulations were observed. In the experiments in which mitochondrial preparations were used, an absolute requirement for ATP was apparent. CoA, especially if added together with cysteine, markedly enhanced fatty acid oxidation. However, the stimulations observed were of approximately the same order of magnitude, whether the mitochondria had been prepared from deficient livers, or from the livers of rats which had received cystine, or choline. Some of these findings are exemplified in Table XV. Thus we are still unable to define the specific deficiency, or deficiencies, which may be directly responsible for the decreased rates of fatty acid oxidation observed in the livers of rats on our low-protein diets.

Furthermore, some other observations made in the course of our more recent experiments have added to the difficulties of interpreting our earlier results. In a number of instances, in which, instead of a 5 or an 8 per cent casein

* Kindly supplied by Dr. E. P. Kennedy.

TABLE XV

Effects of the *in vitro* Addition of CoA and Cysteine on the Oxidation of Palmitate-1-C^{14} in Liver Mitochondria

Rats on diet 26, containing 5 per cent casein. Values per mg N liver mitochondria*

	Rat A (no dietary supplement)		Rat B (choline 0.4% in diet)	
	No additions	CoA and cysteine added	No additions	CoA and cysteine added
Respiratory $C^{14}O_2$: *c/m*	252	1780	541	2378
C^{14}-Acetoacetate: *c/m*	230	2254	350	3142
Total C^{14} from fatty acids: *c/m*	482	4034	891	5510

* 3 hours' incubation in air.

diet, we used a basal diet containing 15 per cent of alpha-protein from soya bean, choline supplementation of the diet did not raise the oxidation of fatty acids *in vitro*.

Similar findings have been reported recently by Fritz.[69] As mentioned before, this author[30] was able to almost exactly duplicate our results on the oxidation of isotopic palmitate, added *in vitro* to the livers of rats on a low-casein diet. However, in subsequent experiments in which a diet containing 10 per cent casein and 10 per cent alpha-protein was used, the livers were quite fatty, but there was no detectable decrease in the ability of the tissue to oxidize palmitate.

A discrepancy between the lipotropic effect of choline and its action on the *in vitro* oxidation of fatty acids is also apparent from the recent results of Bernhard *et al.*[70] These authors maintained rats on a diet containing 8 per cent casein and 12 per cent gelatin, with or without addition of choline: during the last six days of the experiments, all animals received daily injections of a solution of acetate-1-C^{14}. Slices of the livers of these rats were then incubated for two hours, and the amounts and the radioactivity of the tissue fatty acids and of the CO_2 produced during the incubation were determined. There may be some question in the interpretation of the results of such type of experiments, since the slices of the choline-deficient livers had an average content of labeled fatty acids four or five times greater than those of the livers of rats which had received choline. However, the specific activities of the fatty acids in the two types of livers were not much different, nor was a significant difference detectable in the average specific activities of the respiratory CO_2. Assuming that, under the conditions of these experiments, there was no preferential utilization of a more actively metabolized fraction of the newly synthesized fatty acids,[71] and assuming furthermore that the $C^{14}O_2$ produced was derived chiefly from the isotopic fatty acids present in the liver at the beginning of the incubation, the results of Bernhard *et al.*[70] would indicate that fatty acids previously synthesized *in vivo* from acetate were metabolized *in vitro* at essentially similar rates, whether or not choline had been added to the diet. Yet, neutral fat accumulated in much larger amounts in the livers of rats which had been on the diet not supplemented with choline.

It appears today that the decrease in the rate of the oxidation of fatty acids which occurs in the liver of rats on low-protein diets, can be prevented more or less completely by supplementing the diets with choline, or cystine, or tocopherol, as well as by using higher levels of proteins with a minimal content of sulfur amino acid. At present, there is no satisfactory explanation which might include these various findings.

In long range experiments, the possibility of a sparing action of choline by other dietary factors should perhaps be considered. At any rate it would be difficult to explain the enhancement of the *in vitro* oxidation of fatty acids by choline injected terminally, if choline, or a choline derivative, did not have some role, direct or indirect, in the oxidation of fatty acids. Since such a role seems to be clearly demonstrable only in the liver of animals on low-protein diets, one might think also of a possible relationship to the synthesis of proteins, or perhaps more specifically, of lipoproteins, containing lecithins as the lipid moiety. It may be pointed out in this respect that a number of other recent findings suggest that in choline-deficient animals, profound alterations of the metabolism not only of fat, but of proteins also, may develop.[72–75]

The possibility that choline, directly or indirectly, might affect the formation in the liver of lipoproteins which carry most of the fatty acids in plasma, is attractive. Some results reported by Olson and associates in a preliminary form[78] seem to be in line with such a possibility.

Other possibilities can also be visualized, such as an effect of choline on the nonesterified fatty acids of plasma. Indeed, recent data suggest that these fatty acids which represent only a small fraction of the total, may play a considerable role in the transport of fatty acids.

IV. CONCLUSION

It appears today that the evidence for a role of choline in fatty acid oxidation remains restricted to the results of the experiments on preparations of the liver and of a few other

tissues from rats, previously maintained on low-protein diets. In these animals deficiencies of factors other than choline are also present. Administration of choline to such animals enhances the oxidation of fatty acids added to tissues *in vitro*. This effect is generally accompanied by decreases in the amount of fat and by increases in the rate of synthesis and in the level of liver lecithins, thus suggesting that choline may act on fatty acid oxidation because it promotes lecithin formation. However, such relationships are not maintained under all conditions: for instance, low-lecithin levels together with low-fat contents and a high rate of fatty acid oxidation have been found in the livers of protein-deficient rats receiving diethanolamine, a substance which probably interferes with the formation of liver lecithins.

On the other hand, enhancements of fatty acid oxidation *in vitro* similar to those caused by choline administration, have been obtained by supplementing low-protein diets with cystine, or tocopherol, and also by raising the level of dietary protein without substantially increasing the supply of methionine and cystine. Since with diets of the type last mentioned, fat still accumulates in the liver, it appears now that the lipotropic action of choline cannot be explained merely as the result of an enhanced oxidation of fatty acids in the liver.

In earlier and recent experiments *in vivo* with D_2O or with C^{14}-acetate, the amounts of newly synthesized fatty acid present in the liver and in the depots of animals receiving, or not receiving, choline have been compared. The interpretation of the results of this type of experiment is complex and therefore subject to some reservation. Since no evidence was obtained for an increased rate of fatty acid oxidation in the rats fed choline-supplemented diets, such results have been taken as an indication that choline acts by enhancing the mobilization of fatty acids from the liver. It should be pointed out, however, that the two interpretations are not mutually exclusive: indeed, the possibility exists that more than one mechanism is involved in the lipotropic action of choline, and that perhaps the relative importance of these mechanisms may vary under different nutritional conditions. At any rate, since it is unlikely that phospholipids represent a major form either for the transport of fatty acids in plasma, or for the oxidation of fatty acids in the liver, the mode of action of choline in favoring one, or the other, or both processes, remains to be clarified. At the present time, one might only speculate on a relationship between these processes and the synthesis of lecithin-containing lipoproteins in the liver.

Aside from this or similar speculations, the most recent findings have introduced a number of complications and uncertainties in the interpretation of results which a few years ago seemed to clearly suggest an important role of choline in the oxidation of fatty acids in the liver. At the present time, and until we shall be able, first, to define such a role in chemical terms and at the molecular level, and, second, to decide whether, and to what extent, the effects observed in the experiments *in vitro* can be extended to the conditions of the intact animals, I feel that perhaps the title of this paper should be followed by a question mark. In other words, the role of choline in fatty acid oxidation seems to be still a field wide open to future investigations.

REFERENCES

1. Loew, O.: *Biol. Centralbl.* 11:265, 1891.
2. Leathes, J. B.: *Lancet* 1:593, 1909.
3. Artom, C.: *Arch. Fisiol.* 32:57, 1933.
4. Artom, C., Sarzana, G., and Segrè, E.: *Arch. internat. Physiol.* 47:245, 1938.
5. Sinclair, R. G.: *J. Biol. Chem.* 111:515, 1935.
6. Best, C. H., Channon, H. J., and Ridout, J. H.: *J. Physiol.* 81:409, 1934.
7. Perlman, I. and Chaikoff, I. L.: *J. Biol. Chem.* 127:211, 1939.
8. Boxer, G. E. and Stetten, D., Jr.: *J. Biol. Chem.* 153:617, 1944.
9. Artom, C. and Cornatzer, W. E.: *J. Biol. Chem.* 171:779, 1947.
10. Perlman, I., Stillman, N., and Chaikoff, I. L.: *J. Biol. Chem.* 133:651, 1940.
11. Platt, A. P. and Porter, R. R.: *Nature* 160:905, 1947.
12. Artom, C. and Cornatzer, W. E.: *J. Biol. Chem.* 176:949, 1948.
13. Entenman, C., Chaikoff, I. L., and Friedlander, H. D.: *J. Biol. Chem.* 162:111, 1946.
14. Taurog, A., Entenman, C., and Chaikoff, I. L.: *J. Biol. Chem.* 156:385, 1944.
15. Barrett, H. M., Best, C. H., and Ridout, H. J.: *J. Physiol.* 93:367, 1938.

16. Stetten, D., Jr. and Grail, G. F.: *J. Biol. Chem.* 148: 509, 1943.
17. Stetten, D., Jr. and Salcedo, J., Jr.: *J. Biol. Chem.* 156: 27, 1944.
18. Hevesy, G. and Hahn, L.: *Danske Videnskabernes Selskab, Biol. Meddel.* 15: 6, 1940.
19. Entenman, C., Chaikoff, I. L., and Zilversmit, D. B.: *J. Biol. Chem.* 166: 15, 1946.
20. Zilversmit, D. B., Entenman, C., and Chaikoff, I. L.: *J. Biol. Chem.* 176: 193, 1948.
21. Artom, C.: Mechanism of action of lipotropic factors in animals; in *Liver Injury*, Transact. 10th Conf. (ed. by F. W. Hoffbauer). Josiah Macy, Jr. Found., New York, 1951.
22. Weinhouse, S., Millington, R. H., and Volk, M. E.: *J. Biol. Chem.* 185: 191, 1950.
23. Lehninger, A. L.: *J. Biol. Chem.* 157: 363, 1945.
24. Artom, C.: *J. Biol. Chem.* 205: 101, 1953.
25. Jedeikin, L. A. and Weinhouse, S.: *Arch. Biochem.* 50: 134, 1954.
26. Abdon, O. and Börglin, N. E.: *Acta Pharmacol. et Toxicol.* 3: 73, 1947.
27. Potter, V. R. and Klug, H. L.: *Arch. Biochem.* 12: 241, 1947.
28. Lévy, M.: *Arch. des Sciences physiol.* 5: 289, 1951.
29. Lévy, M. and Legrand, J.: *Arch. des Sciences physiol.* 7: 63, 1953.
30. Fritz, I. B.: *Fed. Proc.* 15: 68, 1956.
31. Artom, C.: *J. Biol. Chem.* 213: 681, 1955.
32. Griffith, W. H. and Wade, N. J.: *J. Biol. Chem.* 131: 567, 1939.
33. Keston, H. D., Salcedo, J., Jr., and Stetten, D., Jr.: *J. Nutrition* 29: 171, 1945.
34. Hartroft, W. S., Ridout, J. H., Sellers, E. A., and Best, C. H.: *Proc. Soc. Exper. Biol. & Med.* 81: 384, 1952.
35. Wilgram, G. F., Hartroft, W. S., and Best, C. H.: *Brit. M. J.* 2: 1, 1954.
36. Califano, L.: *Biochem. Ztschr.* 289: 354, 1937.
37. Ennor, A. H.: *Australian J. Exper. Biol. & M. Sc.* 20: 73, 1942.
38. Biagini, C.: *Arch. Soc. Biol.* (Italy) 35: 418, 1951.
39. Rodbell, J. and Hanahan, D. J.: *J. Biol. Chem.* 214: 595, 1955.
40. Marinetti, G. V. and Stotz, E.: *J. Biol. Chem.* 217: 745, 1955.
41. Artom, C.: *Fed. Proc.* 15: 690, 1956.
42. Artom, C. and Fishman, W. H.: *J. Biol. Chem.* 148: 405, 1943.
43. Fishman, W. H. and Artom, C.: *J. Biol. Chem.* 164: 307, 1946.
44. McArthur, C. S., Lucas, C. C., and Best, C. H.: *Biochem. J.* 41: 612, 1947.
45. Strength, D. R., Schaefer, E. A., and Salmon, W. D.: *J. Nutrition* 45: 329, 1951.
46. Stetten, D., Jr. and Grail, G. F.: *J. Biol. Chem.* 144: 175, 1942.
47. Artom, C., Cornatzer, W. E., and Crowder, M.: *J. Biol. Chem.* 180: 459, 1949.
48. Jensen, D., Chaikoff, I. L., and Tarver, H.: *J. Bicl. Chem.* 192: 395, 1951.
49. Feinberg, H., Rubin, L., Hill, R., Entenman, C., and Chaikoff, I. L.: *Science* 120: 317, 1954.
50. Furman, R. H., Norcia, L. N., Robinson, C. W., and Gonzales, I. E.: *Fed. Proc.* 15: 258, 1956.
51. Cornatzer, W. E. and Artom, C.: *J. Biol. Chem.* 178: 775, 1949.
52. Kielley, W. W. and Meyerhof, O.: *J. Biol. Chem.* 183: 391, 1950.
53. Swanson, M. A. and Mitchell, M. C.: *Fed. Proc.* 11: 296, 1952.
54. Nason, A. and Lehman, J. R.: *J. Biol. Chem.* 222: 511, 1956.
55. Lévy, M. and Legrand, J.: *Bull. Soc. Chim. Biol.* 36: 789, 1954.
56. Artom, C.: Formation of phospholipides in animal tissue; in *Phosphorus Metabolism* (ed. by W. D. McElroy and B. Glass). John Hopkins Press, Baltimore, 1952.
57. Beveridge, J. M. R.: *Canad. J. Biochem. Physiol.* 34: 361, 1956.
58. Kennedy, E. P.: *Ann. Rev. Biochem.* 26: 119, 1957.
59. Dawson, R. M. C.: *Biol. Reviews* 32: 188, 1957.
60. Artom, C.: *J. Biol. Chem.* 223: 389, 1956.
61. Schwartz, K. and Foltz, C. M.: *J. Am. Chem. Soc.* 79: 3292, 1957.
62. Olson, R. E. and Dinning, J. S.: *Ann. New York Acad. Sc.* 57: 888, 1954.
63. Olson, R. E., Yang, C. S., Riegel, M., and Stewart, B.: *Fed. Proc.* 14: 447, 1955.
64. Lindan, O. and Work, E.: *Biochem. J.* 55: 554, 1953.
65. Dianzani, M. V. and Viti, I.: *Biochem. J.* 59: 141, 1955.
66. Frunder, H.: *Ztschr. f. Physiol. Chem.* 297: 267, 1954.
67. Christie, G. S. and Judah, J. D.: *Proc. Roy. Soc.* (London) B 142: 241, 1954.
68. Dianzani, M. V.: *Biochem. et Biophys. Acta.* 17: 391, 1955.
69. Fritz, I. B.: *Am. J. Physiol.* 190: 449, 1957.
70. Bernhard, K., Ulbrecht, G., Ulbrecht, M., and Wagner, H.: *Helvet. Physiol. Pharmacol. Acta* 14: 342, 1956.
71. Volk, M. E., Millington, R. H., and Weinhouse, S.: *J. Biol. Chem.* 195: 493, 1952.
72. Engel, R. W.: *J. Nutrition* 36: 739, 1948.
73. Alexander, H. D. and Sauberlich, H. E.: *J. Nutrition* 47: 361, 1952.
74. Alexander, H. D. and Sauberlich, H. E.: *J. Nutrition* 61: 329, 1957.
75. Fischer, M. A. and Garrity, G. C.: *J. Biol. Chem.* 204: 759, 1953.
76. Bernhard, K., Ulbrecht, G., Ulbrecht, M., and Wagner, H.: *Helvet. Chim. Acta* 37: 1439, 1954.
77. Guggenheim, K. and Olson, R. E.: *J. Nutrition* 48: 345, 1952.
78. Olson, R. E., Jablonski, J. R., and Taylor, E.: *Fed. Proc.* 16: 395, 1957.

DISCUSSION

Dr. M. Bates (University of Pittsburgh, Pittsburgh, Pa.): Certainly one of the most interesting problems concerning the fatty livers associated with choline deficiency is the origin of the excessive amounts of fatty acids which accumulate in the liver. The possibility that the choline-containing phospholipids are important as a vehicle in the transport of the fatty acids from the liver has been pretty well eliminated. The evidence is controversial as to whether there is increased fat synthesis in the liver of the choline-deficient animal (K. Bernhard, G. Ulbrecht, M. Ulbrecht, and H. Wagner, *Helv. Chim. Acta* 37: 1439, 1954; K. Guggenheim and R. E. Olson, *J. Nutrition* 48: 345, 1952). Dr. Olson's and Dr. Artom's data would suggest that fat of "normal" specific activity accumulates in the liver of the choline-deficient rat. In addition, the *in vitro* work described by Dr. Artom would indicate that decreased oxidation of fatty acids may also occur in these fatty livers.

Dr. Artom has shown that in choline-deficient animals there is a decrease in the amount of choline-containing phospholipids in the liver and that after an injection of choline there is a marked increase in the amount of this type of phospholipid. Has he tried to correlate the concentration of choline-containing phospholipids in the liver and the amount of fatty acids oxidized? If a correlation did exist it might give some support to the idea that the choline-containing phospholipids are concerned with the oxidation of fatty acids.

Several papers have shown that when a choline deficient liver slice from a rat or dog is incubated with choline there is an increased rate of formation of the choline-containing phospholipids (M. C. Fishler, A. Taurog, I. Perlman, and I. L. Chaikoff, *J. Biol. Chem.* 141: 809, 1941; C. Artom and M. A. Swanson, *J. Biol. Chem.* 193: 473, 1951; N. R. DiLuzio and D. B. Zilversmit, *J. Biol. Chem.* 205: 867, 1953). In the work Dr. Artom described, the *in vitro* addition of choline to liver slices did not result in an increased fatty acid oxidation, and in some cases an inhibition resulted. Wouldn't this suggest that the choline-containing phospholipids are not involved in fatty acid oxidation?

Dr. Kennedy: There is just one comment I would like to make about Dr. Artom's interesting work. Now that the enzymes which catalyze the oxidation of fatty acids have been isolated in pure form in so many laboratories, it would appear that a direct requirement for choline or even for phospholipids in the action of these enzymes is ruled out.

There is one problem remaining, however, and that is the form in which the fatty acid is presented to the mitochondrial fatty-acid oxidase system, whether it is presented as free fatty acid which is activated by ATP plus CoA or whether it is presented as phospholipid or as a neutral fat. It certainly has been well established that the actual form in which the fatty acid is oxidized is the CoA ester and not as phospholipid. In view of the fact that recent studies have shown that the unesterified fatty acids of the blood are the major form in which fats are transported, perhaps it is the unesterified or so-called free fatty acid fraction which is acted upon by the fatty acid oxidase system of the liver cell as well.

Dr. Artom (closing remarks): In reference to Dr. Bates' remarks, it seems to me that our data indicate that a rough parallelism between the amounts of choline-containing phospholipids in the liver and the ability of the tissue to oxidize fatty acids does occur in many, but not in all conditions. Therefore, these findings give little or no convincing evidence for the idea that choline-containing phospholipids are necessarily involved in fatty acid oxidation.

In this respect, Dr. Bates has also pointed out that, while choline administered *in vivo* stimulates both the synthesis of phospholipids and the oxidation of fatty acids, there is some discrepancy in the effects of choline *in vitro*. Choline added *in vitro* does not stimulate fatty acid oxidation, whereas in our earlier experiments on the synthesis of phospholipids the addition of choline to liver slices from protein-deficient rats was partially effective. I believe that in similar experiments on the livers of choline deficient dogs, Dr. Zilversmit found that choline, added *in vitro*, stimulated the formation of lecithins to values even higher than in those found in liver slices from dogs on an adequate diet.

Concerning the suggestion made by Dr. Kennedy, I must admit that I have speculated on the same possibility which Dr. Kennedy has mentioned: that is, that unesterified fatty acids, which are only a very small fraction of the total fatty acids of blood plasma, may represent the form most active metabolically, not only for the transport in blood, but also inside the cells. The data I have shown concerning the distribution of C^{14}-fatty acid at the end of the incubation indicate that in the livers of both normal and choline-deficient rats, the oxidation occurred at the expense of the nonesterified fraction. Possibly the role of choline in this regard is somewhat indirect. Perhaps choline promotes the formation of some lecithin-containing lipoproteins in the liver, which may be important either, as I mentioned, in maintaining the spatial configuration of the enzymes in the mitochondria, or, as Dr. Kennedy has suggested, in favoring the binding of the substrate to the enzymes on the surface of the mitochondria. Moreover, these lipoproteins synthesized in the liver, may represent the form in which fatty acid are carried away from the liver to the depots.

The last point I want to make refers to the effect of cytidine triphosphate and cytidine diphosphate choline. Dr. Kennedy was kind enough to send us some of his compound. Neither cytidine triphosphate or cytidine diphosphate choline had any effect upon the oxidation of fatty acids *in vitro*.

The Role of Choline in the Turnover of Phospholipids

D. B. ZILVERSMIT, PH.D.* AND N. R. DILUZIO, PH.D.†

LET US FIRST consider the nature of the question, "What is the role of choline in the turnover of tissue phospholipids?" If our interest derived solely from the fact that choline is a constituent of a large portion of body phosphatides, one might equally enquire about the role of the phosphate or glycerol moiety in the turnover of phospholipids. Clearly then, the implication is that choline may influence the metabolism of phosphatides in a manner that might explain a more intriguing problem, namely, that of lipotropism and it is this question that we shall consider at the present time.

The recent emphasis on choline, methionine, vitamin B_{12}, and folic acid as lipotropic agents might tend to obscure the fact that lipotropism was discovered by Best and co-workers[1] as an activity of fed phosphatide. It was only after painstaking investigation that it was established that the whole phosphatide molecule was not essential for lipotropic action, but that choline alone could prevent or cure fatty infiltration of the liver.[2] Later, it was discovered that other agents, one or more steps removed from the phosphatide molecule also exhibited lipotropic activity. Nonetheless, the question remained whether these lipotropes acted on fat oxidation or fat transport directly, and whether their lipotropic activity was mediated through the phosphatide molecule. Although no direct evidence has yet been obtained on this problem, there are several reasons why one might wish to adhere to the previously proposed concept that choline removes excessive liver fat by increasing fat oxidation via its action on liver phosphatides. The relation of choline to fat oxidation has been reviewed by Artom in the previous paper.[3] We shall now consider some data relevant to the second part of the hypothesis: "Is the lipotropic action of choline mediated by stimulating the turnover of liver phosphatides?"

One of the earliest investigations in which tracer P^{32} was used, showed that choline increased the incorporation of this label into liver phosphatides of rats maintained on a high-fat, low-protein, choline-deficient diet.[4] At that time phosphatides were considered to be important vehicles for the transport of fatty acids and it was thus logical to study the incorporation of P^{32} into the plasma phosphatides. Figure 1 shows a typical result obtained from a study by Friedlander *et al.*[5] in choline-deficient dogs. There can be no doubt that a single administration of choline to a choline-deficient dog markedly increased the incorporation of inorganic P^{32} into plasma phosphatides. One might be tempted to conclude that in this animal, plasma phosphatide turnover was markedly increased, but a careful study of specific activity relations of liver and plasma phosphatides[6] revealed that the turnover of plasma phosphatides was not increased by choline and that the increased plasma phospholipid specific activity resulted from an increased synthesis of the precursor, namely, liver lecithin (Fig. 2). Here then, was direct proof that if choline diminished liver fat by acting on phosphatide

From the Division of Physiology, University of Tennessee Medical Units, Memphis.

* Professor of Physiology.

† Assistant Professor of Physiology, University of Tennessee Medical Units, Memphis, Tennessee.

Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc., New York, New York.

The work reported in this article was supported by the U. S. Public Health Service (H-1238), Life Insurance Medical Research Fund, and the Lipotropic Research Fund.

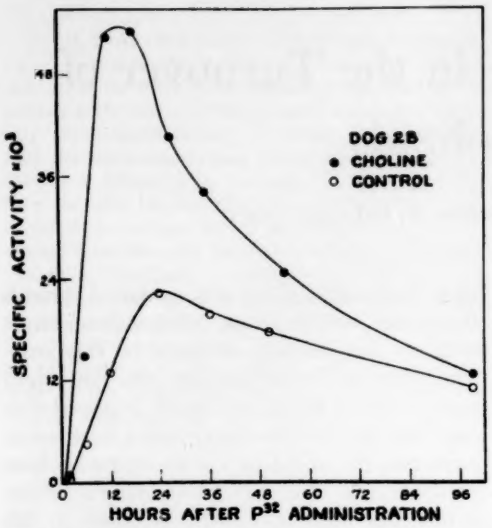


Fig. 1. Specific activity-time relations of plasma phospholipids of the dog after a single injection of P^{32}. The same animal was studied after 14 days on a choline-deficient diet with and without oral choline supplement prior to injection of P^{32}. (Reproduced from Friedlander, H. D., Chaikoff, I. L., and Entenman, C.: *J. Biol. Chem.* 158: 231, 1945; permission of the authors and publishers.)

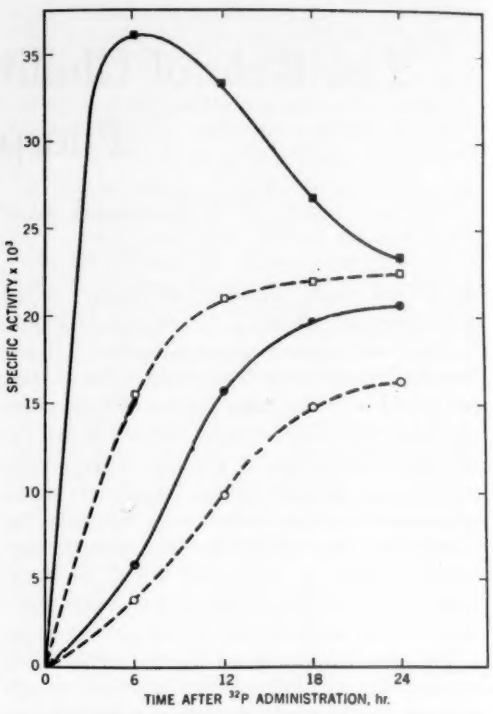


Fig. 2. Specific activity-time relations of plasma and liver choline containing phospholipids in choline-deficient dogs. ○ plasma, □ liver phospholipid specific activity after P^{32}. ● plasma, ■ liver phospholipid specific activity after P^{32} and a single dose of choline chloride. (Reproduced from Entenman, C., Chaikoff, I. L., and Friedlander, H. D.: *J. Biol. Chem.* 162: 111, 1946; permission of the authors and publishe.s.)

metabolism, it did *not* do so by increasing the transport of fatty acids from liver to extrahepatic tissues as an integral part of the plasma phosphatide molecule.

This conclusion was further supported by evidence obtained from hepatectomized animals[7] which demonstrated that plasma phosphatides played little or no role in the transport of fatty acids from one organ to another. If however, choline effects the removal of liver fat through its action on phosphatides, the possibility had to be studied that this action occurred through stimulation of fat oxidation. There is no direct evidence that liver phosphatides participate in fat oxidation but Table I summarizes some of our data on liver phosphatide synthesis in depancreatized[8] and phlorizinized[9] dogs during periods when severe ketosis might be taken to indicate increased utilization in body fat. In both these preparations, the incorporation of P^{32} into liver phosphatides ap-

TABLE I

Liver Phospholipid Synthesis in Diabetic and Phlorizinized Dogs

	Control	Depancreatized	Control	Phlorizinized
Number of dogs	15	10	7	8
Lipid P, mg/g	1.08 ± 0.04	1.13 ± 0.05	1.30 ± 0.10	1.32 ± 0.12
Specific activity*	8.21 ± 0.99	14.9 ± 1.5	4.98 ± 1.07	18.3 ± 1.3

Dogs were injected with P^{32}-phosphate and sacrificed 6 hours later.

* Per cent of the injected P^{32} per g of phospholipid P.

peared to be increased parallel to the increase in fat oxidation. This furnishes at least circumstantial evidence that liver phosphatides may be involved in the catabolism of body fat.

Thus, we may summarize the available evidence as follows: (1) liver phosphatides may well participate in the oxidation of liver fat; (2) a single dose of choline stimulates liver phosphatide synthesis as well as the oxidation of fatty acids; and (3) choline does not increase the transport of liver fat by way of plasma phosphatides. Thus far the evidence supports the view that choline increases liver fat oxidation by stimulating liver phosphatide synthesis but we shall now consider some discordant facts.

First of all there are the experiments with liver slices derived from choline-deficient rats[10] and dogs.[11] As shown in Table II, phosphatide synthesis in the slices of dog liver incubated with P^{32} is depressed as might be expected if choline were essential for the normal turnover of liver phosphatides. Similar results were obtained in rat-liver slices.[10] The addition of choline to the incubation medium had no effect on phosphatide synthesis of normal liver slices or on slices from animals supplemented with dietary choline but greatly accelerated the incorporation of P^{32} into the choline-containing phosphatides of the choline-deficient liver slice. Thus, if choline were to act on fat catabolism merely by increasing liver phosphatide synthesis one might reasonably expect that whenever choline stimulated liver phosphatide turnover it would also increase the rate of fat oxidation. This is clearly not the case. Artom[12] showed that although injected choline stimulates fat oxidation by liver slices, the addition of choline to the incubating medium itself had no such effect. It is possible, of course, that fat oxidation in the liver slice is limited by factors other than the availability of phosphatides, but whatever the explanation, it is clear that stimulation of liver phosphatide synthesis is not the only prerequisite for an increase in fat oxidation.

Another disturbing aspect of the situation appeared when the effect of a single dose of choline on the synthesis of liver phosphatides was compared with the effects of chronic supplements of this lipotropic agent. In one experiment, rabbits weighing 1.5 to 3 kg were placed on four different diets: (1) Purina chow; (2) high-fat, low-protein, choline-deficient diet;* (3) the same diet with daily choline supplements, and (4) the same diet with a single intravenous injection of choline at the end of the 14-day experimental period. Radioactive phosphate was administered to all animals to measure liver phosphatide synthesis. The rabbit was originally chosen for this experiment to study the relationship between choline deficiency, phosphatide metabolism, and atherosclerosis.[13] However, it appears that the rabbit may be particularly suited for the study of choline deficiency since this animal converts the methyl of methionine to choline very poorly.[14] The results in Table III indicate that although the experimental animals were maintained on a low-protein, high-fat, choline-supplemented diet, their liver phosphatide concentration or synthesis did not differ from the chow-fed con-

TABLE II

Effect of Choline on Phospholipid Metabolism of Dog Liver Slices

Diet	Choline-containing phospholipids *mg/g*	P converted choline-containing phospholipid/hr/g	
		Without choline	With choline
Purina chow	0.67 ± 0.02	7.6 ± 0.67	8.3 ± 0.91
High-fat, low-protein, plus 1% choline (21 days)	0.44 ± 0.02	3.7 ± 0.32	4.7 ± 0.46
High-fat, low-protein, choline-deficient (21 days)	0.35 ± 0.01	4.0 ± 0.32	18.4 ± 1.17

Dog liver slices were incubated for 1 hour with P^{32}-phosphate in the presence and absence of choline chloride, 100 mg per 100 ml.

* The diet consisted of 38 g lard, 8 g casein, 44 g sucrose, 3 g of brewer's yeast, 5 g of Cowgill's salt mixture, and 2 ml of cod liver oil.

TABLE III

Liver Phosphatide Metabolism of Rabbits

	Purina chow (1)	High-fat–low-protein diet 14 days		
		Daily oral choline (2)	No treatment (3)	Single dose intravenous choline (4)
Number of animals	9	6	5	6
Lipid P, mg P/g	1.06 ± 0.10	1.17 ± 0.04	1.25 ± 0.09	1.16 ± 0.12
Specific activity*	12.4 ± 1.2	13.5 ± 1.9	22.6 ± 2.1	38.7 ± 5.1

Rabbits were sacrificed 6 hours after administration of P^{32}-phosphate.

* Per cent of the injected P^{32} per g of phospholipid P.

trols as long as choline was present in the diet (compare columns 1 and 2). Apparently the presence of large amounts of fat or little protein *per se* did not affect liver phosphatide metabolism. However, when choline was *withdrawn* from the diet, liver phosphatide synthesis, as measured by the incorporation of P^{32}, nearly doubled. A further marked increase in liver phosphatide P^{32} took place (columns 3 and 4) when a single dose of choline was given to the choline-deficient animals simultaneous with the injection of P^{32}. Thus, the discussion of choline action requires a sharp differentiation between the effects of a single dose of choline and that of daily choline supplements. A somewhat similar result was obtained by Cayer and Cornatzer[15] in patients with liver disease who showed an above normal incorporation of P^{32} into plasma phosphatides the first time they received choline but failed to show any increase after a period of choline therapy.

To further evaluate these findings similar studies were undertaken in the dog (Table IV). Although liver phospholipid concentrations in the deficient animals decreased significantly, the P^{32} data are essentially similar to the data previously obtained in the rabbit. They show that incorporation of inorganic phosphate into liver phosphatides in the choline-deficient animals is greater than that of the choline-supplemented controls. While a single dose of choline in the dog resulted in enhanced liver phosphatide P^{32} formation, the daily administration of choline decreased this process below that of the choline-deficient animals. At the present time we cannot reach a satisfactory explanation for the results which we have obtained. It appears that the stimulation of liver phosphatide P^{32} synthesis by choline occurs only when the supply of dietary choline is the limiting factor in the series of reactions leading to phosphatide formation. In the animal which has been treated for some time with choline, and in which liver fat concentrations have decreased to normal levels, one might assume that factors such as the supply of triglycerides become limiting and keep the rate of phosphatide synthesis down at the level observed in animals fed low-fat commercial chow.

An alternative explanation might be based on the observation that free choline normally is removed from the bloodstream very rapidly.[16] The two processes which presumably are most important in this removal are the oxidation of choline by the aid of choline oxidase and the incorporation of choline into tissue lecithin.

TABLE IV

Liver Phospholipid Metabolism in Dogs

	Purina chow	High-fat–low-protein diet 21 days		
		Daily oral choline	No treatment	Single oral dose choline
Number of animals	12	6	8	6
Lipid P, mg/g	1.19 ± 0.04	0.97 ± 0.05	0.88 ± 0.05	1.09 ± 0.10
Specific activity*	10.2 ± 0.87	12.2 ± 1.55	19.4 ± 1.98	25.4 ± 3.0

Dogs were sacrificed 6 hours after administration of P^{32}-phosphate.

* Per cent of the injected P^{32} per g of phospholipid P.

Since in the choline-deficient animal choline oxidase activity in the liver is sharply reduced[17] one might assume that in the deficient animal more choline would remain available for incorporation into phosphatides.

It is understood, of course, that one must be careful in concluding that the increased incorporation of P^{32} into a liver phosphatide fraction necessarily represents increased synthesis or increased turnover of liver phosphatide. While no differences in the specific activities of liver inorganic and organic acid-soluble phosphates were noted in the various experimental groups, it is quite possible that a single dose of choline administered to a choline-deficient animal might stimulate the formation of a specific phosphatide precursor or by some other means increase the specific activity of a phosphorylated intermediate such as cytidine diphosphate choline. To differentiate between the stimulation of phosphatide synthesis proper and the increased labeling of some phosphatide precursor one must measure the specific activity-time relations of the phosphatide fraction and its immediate precursor. Such an analysis may well clarify the differences between single and continued choline administrations and supply the missing links in our present knowledge about the effect of dietary choline on liver phosphatide synthesis.

It is obvious, however, from the results which we have presented, that the relationship of phosphatides to the lipotropic activity of choline is still obscure. The available evidence indicates that there is no simple relation between the rate of lipid phosphorylation in liver and the amount of lipotropic factors in the diet, or the extent of lipotropic activity from a single dose of choline or methionine (see also Horning and Eckstein[18]). The observed stimulation of liver phosphatide metabolism by a single dose of choline may turn out to be unrelated to lipotropic activity. Yet the fact that choline is an integral part of the liver lecithin molecule, and that under some conditions choline stimulates liver lecithin synthesis, presents enough circumstantial evidence to discourage the investigator from rejecting a hypothetical causal relationship between lipotropism and phosphatide metabolism.

SUMMARY

The effect of choline and choline deficiency on liver phosphatide metabolism has been studied in various species. Evidence to date indicates that a single dose of choline administered to choline-deficient animals increases the oxidation of liver fat as well as the synthesis of liver phosphatide P^{32}. On the other hand, the addition of choline to liver slices derived from choline-deficient animals promotes the incorporation of P^{32} into the phosphatide molecules without stimulating fat oxidation. In addition, it was observed that in the rabbit and dog the rate of incorporation of P^{32} into liver phosphatides is greater during periods of choline deficiency than during daily supplementation of the diet with 1 per cent choline. The implications of these findings are discussed in reference to the mechanism whereby choline prevents or cures fatty livers.

REFERENCES

1. Best, C. H., Hershey, J. M., and Huntsman, M. E.: The effect of lecithine on fat deposition in the liver of the normal rat. *J. Physiol.* 75: 56, 1932.
2. Best, C. H. and Huntsman, M. E.: The effects of the components of lecithine upon deposition of fat in the liver. *J. Physiol.* 75: 405, 1932.
3. Artom, C.: Role of choline in the hepatic oxidation of fat. Am. J. Clin. Nutrition 6: 221, 1958.
4. Perlman, I. and Chaikoff, I. L.: Radioactive phosphorus as an indicator of phospholipid metabolism. V. On the mechanism of action of choline upon the liver of the fat-fed rat. *J. Biol. Chem.* 127: 211, 1939.
5. Friedlander, H. D., Chaikoff, I. L., and Entenman, C.: The effect of ingested choline on the turnover of plasma phospholipides. *J. Biol. Chem.* 158: 231, 1945.
6. Entenman, C., Chaikoff, I. L., and Friedlander, H. D.: The influence of ingested choline upon choline-containing and non-choline containing phospholipids of the liver as measured by radioactive phosphorus. *J. Biol. Chem.* 162: 111, 1946.
7. Entenman, C., Chaikoff, I. L., and Zilversmit, D. B.: Removal of plasma phospholipides as a function of the liver: The effect of exclusion of the liver on the turnover rate of plasma phospholipides as measured with radioactive phosphorus. *J. Biol. Chem.* 166: 15, 1946.
8. Zilversmit, D. B. and DiLuzio, N. R.: Synthesis of phospholipides in diabetic dogs. *J. Biol. Chem.* 194: 673, 1952.
9. DiLuzio, N. R. and Zilversmit, D. B.: Turnover

of liver and plasma phospholipides of phlorizinized dogs. *Am. J. Physiol.* 170: 472, 1952.

10. ARTOM, C. and SWANSON, M. A.: Incorporation of labeled phosphate into the lipides of liver slices. *J. Biol. Chem.* 193: 473, 1951.
11. DILUZIO, N. R. and ZILVERSMIT, D. B.: The effect of choline on phospholipide synthesis in dog liver slices. *J. Biol. Chem.* 205: 867, 1953.
12. ARTOM, C.: Role of choline in the oxidation of fatty acids by the liver. *J. Biol. Chem.* 205: 101, 1953.
13. DILUZIO, N. R. and ZILVERSMIT, D. B.: Effect of choline on phosphatide metabolism of choline-deficient and cholesterol-fed rabbits. *Proc. Soc. Exper. Biol. & Med.* 92: 454, 1956.
14. HOVE, E. L., COPELAND, D. H., and SALMON, W. D.: Choline deficiency in the rabbit. *J. Nutrition* 53: 377, 1954.
15. CAYER, D. and CORNATZER, W. E.: The effects of choline and methionine on phospholipide formation in patients with liver disease as measured by radioactive phosphorus. *Science* 109: 613, 1949.
16. BLIGH, J.: The role of the liver and the kidneys in the maintenance of the level of free choline in plasma. *J. Physiol.* 120: 53, 1953.
17. HUMOLLER, F. L. and ZIMMERMAN, H. J.: Relation of choline oxidase activity to dietary fatty livers. *Am. J. Physiol.* 174: 199, 1953.
18. HORNING, M. G. and ECKSTEIN, H. C.: Influence of choline and methionine on phospholipid activity and total lipid content of livers of young white rats. *J. Biol. Chem.* 166: 711, 1946.

DISCUSSION

Dr. W. Wells (University of Pittsburgh, Pittsburgh, Pennsylvania): Dr. Zilversmit in his excellent presentation has pointed out again the confusing nature of the role of choline in the phospholipid turnover. The importance of the administration of choline was pointed out as well as the importance of the level of fat in the diet. I would like Dr. Zilversmit to comment (1) on the effect of age. That is, as I understand it, in the adult animal there is little effect of choline on the phospholipid turnover, whereas in a weanling animal the effects of dietary choline toward increasing the phospholipid concentration and turnover have been shown and (2) on an earlier finding of his that the turnover of plasma cephalin, lecithin, and sphingomyelin were significantly increased in concentration in animals that had been fed cholesterol. I believe these were rabbits. In other words, is there any connection between cholesterol absorption and the phospholipids? It has been shown that choline has very little effect on the absorption of cholesterol. The work of Mann and the Harvard group suggests that atherosclerosis cannot be induced in monkeys which have been fed a low-methionine diet or a low sulfur-containing diet unless choline is added to that diet.

Dr. Kennedy: I should like to make one very brief comment, and that is strongly to reinforce the suggestion that Dr. Zilversmit made that in a P^{32} experiment of this kind one should compare the specific activity of the lecithin with its immediate precursor rather than with the total acid-soluble phosphorus fraction, because the cytidine diphosphate choline content of liver is so low that it is a very minute fraction of the total acid-soluble phosphorus fraction. Perhaps the otherwise very puzzling results that Dr. Zilversmit reports could be explained on the basis of the higher specific activity of the CDP-choline in some experiments.

Dr. W. Hartroft (Washington University, St. Louis, Missouri): I would like to ask if from the experiments with dogs and rabbits you have any data on the effect of a single injection of choline into control animals receiving the basal diet supplemented with choline during the entire experimental period? Results might clarify what part (if any) of the effects observed from this procedure in choline-deficient animals was the result of a pharmacologic or physiologic action of the injected choline.

Dr. Artom: I was interested in the difference in the results obtained by Dr. Zilversmit, when choline was given in one dose, or when it was added to the diet of his rabbits. We have observed a similar difference in our study of the action of diethanolamine on the incorporation of P^{32} into the lipids of rat liver. Given in one single dose, this analogue of ethanolamine stimulated the synthesis of cephalins and also of lecithins. On the other hand, as I mentioned in my paper, when diethanolamine was mixed with the diet, and therefore absorbed in small repeated doses over a period of several days, it acted as an inhibitor of the synthesis of lecithins.

Dr. Herndon: Isn't the effect of giving diethanolamine pharmacologic rather than physiologic?

Dr. Olson: During the last several years we have been engaged in a study of the lipoproteins of liver (*Chemistry of Lipides as Related to Atherosclerosis*, pp. 108–111, Thomas, Springfield, Illinois, 1958). As has already been mentioned the lipids of liver and the lipoproteins of which they are a part are contained largely in the particulates (nuclei, mitochondria, microsomes, and membranes). The cytoplasm is essentially free of these entities. These lipoproteins (and I speak of the group collectively since our studies thus far have been confined to whole homogenates made with a Waring blendor) appear to be quite different from those in the serum. They are more unstable, of high density (flotate only at 1.21 with a $-S$ value of 40 to 60), of apparently different frictional ratio than the high density alpha's of serum, and contain nitrogen, total lipid, phospholipid, and cholesterol in the approximate ratio of 4:40:20:2. Of course, these are rough approximations of the properties of this heterogeneous group of compounds and much more work must be done with the fractions from individual particulates before our knowledge of this interesting group of lipoproteins is complete. We must remember, however, that when we speak of lipid metabolism in the liver we are talking

about the biochemical activity of the lipids in the particulates under normal conditions. The accumulation of neutral fat in the cytoplasm in choline deficiency probably results from relative "overproduction" of triglyceride by the mitochondria under conditions which prevent it from being moved into the serum at the proper rates. The subsequent effects of this triglyceride upon the integrity of the particulates may be related to a biophysical rather than a biochemical effect by which some of the lipids of the particulates are "solubilized" in the sea of neutral fat. Dianzani and Viti (*Biochem. J.* 59: 141, 1955) have reported the transfer of cytochrome C, from mitochondria to cytoplasm in the fatty liver produced by carbon tetrachloride. Finally, although the role of the lipids in the lipoprotein micelle which is found in the organized mitochondrion has been thought to represent merely a "glue" to hold various hydrogen transport and other enzymes in proper apposition, it is apparent from a recent paper by Marinetti *et al.* (*J. Biol. Chem.* 229: 1027, 1957) that the lipids of cytochrome b-cytochrome c_1 complex may participate in electron transport.

Dr. Zilversmit (closing remarks): We have no data ourselves on the effect of age on phospholipid turnover and the effect of choline thereon. There are numerous studies on the phospholipid level of choline-treated animals at different ages and I would be interested in hearing of studies on the turnover of phospholipid.

As far as the relation of choline to cholesterol is concerned, I am afraid I have very little to offer except to say that we were interested in this question. This was one reason why we performed the rabbit experiment since, as you know, in the rabbit cholesterol feeding produces a pronounced fatty liver and lesions in the aorta. We thought that since a single dose of choline has a stimulating effect on liver phospholipid formation, it might have an effect on other organs, such as the aorta as well. We were highly disappointed to learn that a single dose of choline in either a choline-deficient animal or in a cholesterol fed rabbit has no effect whatsoever on the synthesis of phospholipids (*Proc. Soc. Exper. Biol. & Med.* 92: 454, 1956).

There is some disagreement between our laboratory and Chaikoff's laboratory (*J. Biol. Chem.* 128: 735, 1939) and the group of Clement in France (*Compt. Rend.* 236: 412, 1953) on the effect of cholesterol on liver phospholipid synthesis. Both Clement and Chaikoff claim that in the rat liver phospholipid synthesis is greatly inhibited by cholesterol feeding. We have never exactly reproduced this experiment but we have fed some cholesterol to rats and found very little, if any, effect. On the other hand in the rabbit, cholesterol feeding causes a marked increase in phospholipid synthesis.

With regard to Dr. Hartroft's question, we have done the experiment he suggested, and the single dose of choline had no effect in animals which had been supplemented; neither had it any effect in animals which were on a chow diet. I think it has been also shown by Cornatzer and others in patients who have been treated with choline or methionine for a while that a single dose of choline has no effect on phospholipid formation. This comment also would be pertinent to the drug action. Even though there might still be some nonspecific type of effect, say upon oxygen consumption, which we have not measured, the fact remains that these nonspecific effects do not occur in these other preparations and this possibility is eliminated.

Nutritional Fatty Livers in Rats

A. E. HARPER, PH.D*

THE EXTENSIVE literature on nutritional factors that affect the deposition of liver fat has been reviewed, either directly or indirectly, in such detail during the past 15 years[1–15] that one wonders how much another discussion of the subject can add to what has already been said. In an attempt to avoid presenting simply a review of reviews, emphasis has been placed primarily on the investigations on the lipotropic action of protein, in which we at the University of Wisconsin have participated. These have resulted in an understanding of some of the previously conflicting results on this subject. Emphasis has also been placed on the relationships now apparent among the main groups of lipotropic factors.

USE OF THE RAT IN LIPOTROPIC STUDIES

The occurrence of fatty livers in experimental animals was described as early as 1889 by Mering and Minkowski who were studying diabetes in the depancreatized dog. It was as a direct result of the further studies on diabetes[16] that the nutritional significance of fatty livers was recognized. When insulin became available, depancreatized dogs could be maintained for long periods of time on insulin. Under such conditions they were found to develop severe fatty livers[17–19] which could be prevented by adding raw beef pancreas to the diet.[20] Subsequently, Hershey[20] and Hershey and Soskin[21] undertook experiments based on the assumption that the depancreatized dog suffered from a defect in fat metabolism, and showed that the addition of lecithin to the diet prevented the excessive accumulation of fat in the liver. In a continuation of these studies at Toronto a test diet high in saturated fats was developed and the rat was used as the experimental animal. It was demonstrated that lecithin[22] and later choline[23,24] one of the hydrolytic products of lecithin, successfully prevented fatty infiltration of the liver in this species as well. This marked the beginning of studies on fatty livers in the rat; studies which have contributed greatly to our understanding of the complex relationships that exist among the various lipotropic factors.

The fact that choline was not unique in its ability to prevent excessive fat accumulation in the livers of rats fed on hypolipotropic diets became apparent when Best and Huntsman[24] found that betaine exerted a lipotropic effect. They also observed that casein prevented the expected rise in the level of liver fat in rats that were transferred from hypolipotropic diets to diets consisting only of sucrose.[25] These observations led to two distinct but related series of investigations; one, the study of the lipotropic effect of protein; the other, the study of the metabolism of methyl groups and the relationship between methyl metabolism and lipotropic activity.

THE ROLE OF PROTEIN

Initially the Toronto group[25] suggested that the lipotropic effect of casein might be due to impurities or to betaines arising from the casein. However, in the meantime, Channon and Wilkinson[26] had demonstrated that the lipotropic effect of protein was proportional to the level of protein in the diet. They suggested that certain of the amino acids in casein might give rise to choline or betaine in the tissues, a suggestion that was discussed by Best and Channon.[27] A number of proteins were subsequently studied in both the Toronto[28] and the Liverpool laboratories[29] and marked differ-

From the Dept. of Biochemistry, University of Wisconsin.

* Assistant Professor of Biochemistry.

Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc., New York, New York.

ences in their lipotropic effects were noted.

During this time Channon and associates[30] observed that the level of fat in the livers of rats fed on diets containing 5 per cent of casein and 0.1 to 0.2 per cent of choline was about double the value generally found when the diets contained high levels of casein. This suggested that casein exerted a lipotropic action beyond that of choline and led them to undertake a methodical study of the effects of individual amino acids. They observed that cystine exerted an "antilipotropic" effect which could be reversed by feeding additional casein; however, when they tested a number of other amino acids individually all were without lipotropic effect.[31] These observations stimulated interest in the sulfur amino acids and the following year Tucker and Eckstein[32] confirmed the observations of Beeston and Channon[31] on cystine and found that methionine was effective in preventing fatty livers in rats consuming choline-deficient diets. The lipotropic effect of methionine was amply confirmed in several laboratories.[33–35]

Both Tucker and Eckstein[36] and Channon and associates[29] in subsequent studies emphasized the importance of the cystine:methionine balance in accounting for the lipotropic action of protein in rats fed on diets deficient in choline. Certain discrepancies in their results, however, led them to suggest that other amino acids might be of some significance. In 1940 Best and Ridout[34] compared the effectiveness of a 30 per cent casein diet with that of the cystine and methionine it contained and concluded that, since the casein was more effective, other amino acids or some impurity carried by the casein probably exerted a lipotropic action. Channon, and associates[37,38] extended this work and came to similar conclusions but were unable to demonstrate any lipotropic action of 14 individual amino acids. The Ann Arbor group, on the other hand, were able to account for the lipotropic activity of casein in their later experiments entirely on the basis of the dietary content of cystine and methionine,[39,40] and these observations were confirmed.[41,42]

Their results indicated, however, that methionine was less effective as a lipotropic agent in young growing rats than in adult rats, and was also less effective in rats fed on high-protein diets than in those fed on low-protein diets.[40,41] This suggested that the effectiveness of methionine in preventing the accumulation of liver fat decreased when the demand for amino acids for protein synthesis increased. In an attempt to resolve some of the discrepancies regarding the comparative value of the sulfur amino acids and protein as lipotropic agents Beveridge, Lucas and O'Grady[43,44] undertook experiments in which the levels of all of the essential amino acids were carefully controlled. Their results showed clearly that free methionine and casein containing equivalent methionine were equally effective in preventing an accumulation of liver fat when the amounts of essential amino acids in the diets were equalized. With an inadequate diet, however, methionine proved more effective than casein containing equivalent methionine, presumably because little methionine was required for growth in the absence of additional casein. With a more adequate diet, on the other hand, casein proved more effective than equivalent free methionine, which suggested again that protein exerted a lipotropic effect beyond that of the methionine it contained.

The "antilipotropic" effect of cystine had, during this time, been investigated by Griffith and Mulford.[45,46] They had shown that liver fat values were affected by food intake and suggested that the "antilipotropic" effect of cystine could be explained as a result of the growth stimulation produced when cystine was added to sulfur-amino acid-deficient diets. They concluded that when the nutritive value of a diet was improved the increased demand for methionine for growth reduced the quantity available for lipotropic activity and that this in turn caused an increase in the deposition of liver fat. This explanation eliminated the need to postulate a toxic effect of cystine and accounted for the previously difficult-to-reconcile observations, that the "antilipotropic" effect of cystine was not proportional to the amount fed and was not evident when adequate protein was provided in the diet.

SPECIFIC AMINO ACID EFFECTS

About this time, during studies on niacin-

tryptophan relationships, it was noted at Wisconsin[47] and at Georgia[48] that the livers of rats fed on low-protein diets containing choline appeared fatty. These observations and later observations at Wisconsin[49,50] again suggested that amino acids other than methionine might influence the deposition of liver fat and that the effect could not be attributed to a choline-sparing action. In subsequent studies Singal and associates[51] demonstrated that partial deficiencies of either threonine or lysine led to some accumulation of liver fats in rats receiving what were considered to be adequate quantities of choline. Similar observations regarding threonine were reported from our laboratories.[52] It has been shown since that fat accumulates to the extent of about 10 per cent on a fresh weight basis in the livers of young and mature rats fed on a variety of low-protein diets containing adequate amounts of choline.[53–65] Some of these effects are summarized in Table I.

TABLE I

Effect of Additional Protein and Amino Acids on the Deposition of Liver Fat in Rats Fed on Low-Protein Diets Containing 0.15% of Choline Chloride

Protein source, and %	Supplement, and %	Rate of gain, g/wk	Liver fat % dry wt
9 Casein*	—	14	47
9 Casein	—	14	31
9 Casein	0.36 DL-threonine	21	14
9 Casein	2 Casein	26	14
8 Egg albumin	—	8	29
8 Egg albumin	0.25 L-histidine.HCl		
	0.36 DL-threonine		
	0.5 L-lysine.HCl	16	18
89 Rice (5.9 protein)	—	9	29
	0.4 DL-threonine		
	0.5 L-lysine.HCl	20	13

* Choline omitted.

At first these observations were considered to be only remotely related to the earlier studies on the lipotropic effect of protein. It became evident, however, when Eckstein[66] was again unable to demonstrate any lipotropic effect of individual amino acids other than methionine, that choline-deficient diets had been used almost exclusively in the many studies on this subject. It was also evident that, in the experiments in which the lipotropic effect of protein was completely accounted for by its content of methionine, the protein content of the diets was probably too low to provide sufficient methionine to produce a maximal lipotropic response.[67] Also, liver fat values approaching 10 per cent on a fresh weight basis had been considered normal and a reduction from 10 to 5 per cent was not considered significant despite the suggestion of Channon *et al.*[30] Further, differences of this order were obtained in studies in which protein was found to exert a lipotropic effect beyond that of the methionine it contained[30,34,44] and similar reductions were observed when threonine was added to a threonine-deficient diet[52] as can be seen in Table I. These observations suggested that there were *two* distinct lipotropic effects of protein; one, the choline-sparing effect of methionine[32] and, two, the effects of other essential amino acids, such as threonine, in preventing the less extensive accumulation of liver fat which occurred when protein was provided in inadequate amounts.[51–65]

That these conclusions were correct was demonstrated experimentally by comparing the lipotropic activity of methionine, choline, protein and threonine under conditions in which each one became limiting in turn[68–70] as shown in Table II.

As a result of histologic studies there was further evidence that deficiencies of amino acids other than methionine cause a defect in liver metabolism which is different from that produced by choline deficiency. In contrast to the centrolobular distribution of fat observed in the livers of choline-deficient rats, "only occasionally (was) the fatty infiltration most severe around the central vein of the lobule" when protein or amino acid deficiencies were responsible for fatty infiltration.[71] This periportal distribution of liver fat is observed when low-protein diets containing natural products or purified proteins are fed and although the total quantity of fat in the liver is less than that

observed in choline deficiency many cells contain very large amounts of fat.[59–63] The similarity of this lesion to that observed in kwashiorkor in humans has been suggested.[59,60,63]

There is still some question regarding the specificity of the effects of essential amino acid deficiencies in inducing fatty livers. Without doubt conditions can be adjusted to produce fatty livers which respond specifically to threonine, lysine and tryptophan[51,52,62] and it is quite probable, in view of the failure in certain cases to obtain satisfactory responses to individual amino acid supplements, that other amino acid deficiencies may give similar effects.[54,57,65] Not all low-protein diets, however, cause fatty livers to develop. The development in at least one case appears to depend upon the amino acid balance of the diet[72] as shown in Table III. Also, normal liver fat values may be obtained in some cases when high-protein diets deficient in a single amino acid are fed.* This suggests that the total nitrogen level of the diet as well as the balance of amino acids may influence the effects of amino acid deficiencies and, indeed, it has been noted that certain non-essential amino acids in rather high levels may cause some reduction in the level of fat in the livers of rats consuming protein-deficient diets that contain choline.[68,73]

TABLE II

Lipotropic Activity of Protein, Methionine, Threonine and Choline in Rats Fed on Low-Protein Diets

Casein %	DL-methionine %	Choline chloride %	Supplement	Deficiency	Liver fat % dry wt
9	0.3	—	—	Protein and choline	40
9	0.3	0.15	—	Protein	27
9	0.3	—	6 gelatin	Choline	32
9	0.3	0.15	6 gelatin	—	16
9	1.0	—	—	Protein	28
18	0.7	—	—	—	14
9	0.3	—	0.36 DL-threonine	Choline	45
9	1.0	—	0.36 DL-threonine	—	17
40	—	—	—	—	18

TABLE III

Effect of Amino Acid Balance on the Deposition of Liver Fat in Rats Fed on a Low-Protein Diet

Casein %	DL-methionine %	DL-threonine %	Rate of gain g/wk	Liver fat % dry wt
9	—	—	10	14
9	—	0.36	9	12
9	0.3	—	12	31
9	0.3	0.36	19	16

TRANSMETHYLATION, METHIONINE AND BETAINE

Now to return to the chain of events which ensued from the observations that the methyl-containing compounds betaine[24] and methionine[32] exerted marked lipotropic effects in rats fed on choline-deficient diets. At about the same time as the lipotropic activity of methionine was demonstrated the fact that it was an indispensable amino acid was also discovered.[74] These observations led to intensive investigations of the metabolism of the sulfur-containing amino acids during which it was found that homocystine could replace methionine in the diet of young rats as long as choline was provided.[75] This suggested that the methyl group of choline might be transferred to homocystine. It then became evident that the reverse process, the transfer of the methyl group of methionine to a choline-precursor might also occur.[76,77] That this was the case was established when it was found that the methyl group of the choline isolated from the tissues of rats

* H. E. Sauberlich, personal communication; A. E. Harper, unpublished results.

fed on choline-deficient diets containing methionine labeled with deuterium in the methyl group, contained close to the theoretic amount of deuterium that would have been expected had all the methyl groups come from methionine.[78] Thus, the lipotropic activity of methionine could be attributed to its ability to contribute methyl groups for choline synthesis. Betaine was also shown to provide methyl groups for the synthesis of methionine[75,79] and since the methyl group of methionine entered the choline molecule it was evident that that of betaine could do likewise.

The metabolism of methyl groups was now actively investigated in a number of laboratories and the developments have been comprehensively reviewed.[10,12,80] Without going into detail, it became evident that there were two types of methylation reactions;[81] one, in which methionine in an active form served as the methyl donor and guanidinoacetic acid or nicotinamide serve as acceptors; the other, in which betaine and certain thetins served as methyl donors and homocystine or homocysteine as acceptors. Since both methionine and betaine serve as precursors of choline *in vivo*, it has been assumed that the methyl groups of these compounds can be transferred to acceptors such as ethanolamine, monomethylethanolamine or dimethylethanolamine in the animal body. There are species differences with regard to the efficiency of different acceptors but in the rat, ethanolamine, which can be synthesized by the tissues from glycine and serine serves this purpose. Many of the relationships among these compounds have not been worked out in detail but the evidence suggests that betaine itself is not reduced in the animal body to give choline directly.[77,82] It has been more generally accepted that a methyl group of betaine is transferred to homocysteine, which can arise in the body from methionine. This transfer would then regenerate methionine which, upon conversion to the active form,[83] could in turn transfer the methyl group received from betaine to other methyl acceptors.[12,80] Stekol[84] has outlined a pathway by which dimethylethanolamine may be formed independently of the direct transmethylation reactions, however, transmethylation from methionine would still presumably provide the third methyl group for choline synthesis.

The lipotropic activities of methionine and betaine are therefore accounted for by their roles in providing methyl groups for the synthesis of choline which is, in the final analysis, the compound that is required for the control of liver fat deposition. Although it has been emphasized before,[5,6] it is probably not out of place to re-emphasize the fact that arsenocholine[85] and the triethylhomologue of choline,[86] which are not methyl donors, can protect animals on choline-deficient diets against fatty livers. *Thus it is evident that fatty livers do not result directly from a methyl deficiency, as is sometimes said, but from a choline deficiency.* (There is also evidence that triethylcholine may act as a choline antagonist[87,88] so its role is not completely clear.)

BIOSYNTHESIS OF METHYL GROUPS. VITAMIN B_{12}

During the course of the studies that led to our present understanding of the phenomenon of transmethylation certain anomalous results were obtained which opened up another facet of the metabolism of methyl groups and led to the discovery that the most recently characterized members of the vitamin B complex also played a role in decreasing the severity of certain types of fatty livers.[7–10,12,14,80]

The ease with which choline and methionine deficiencies could be produced in the rat cast doubt for a long time upon the possibility that this animal could synthesize methyl groups. However, during the period in which transmethylation was being actively studied, it was observed by du Vigneaud and associates[75] that the occasional animal grew slowly on a diet containing homocystine but no source of methyl groups. Then Bennett, Medes and Toennies[89,90] found that such an effect could be produced with some regularity. They suggested that unknown dietary factors might facilitate the synthesis of methyl groups from unknown precursors. It was subsequently demonstrated that such a synthesis did occur[91–94] and that vitamin B_{12} was a component of the system required for it in both the rat[94,95] and the chick.[96,97] The route of synthesis has been

studied extensively and many of the details have been elucidated.[80,98]

The significance of vitamin B_{12} and the synthesis of methyl groups in the control of liver fat deposition have been the subject of a number of investigations,[12,45,88,99–101] of which only a few can be mentioned. Stekol[84] has outlined much of the evidence and has discussed the possibility that folic acid may also be involved in certain of the pathways. Bennet and associates[99] demonstrated that a mixture of vitamin B_{12} and homocystine was as effective as equivalent methionine in preventing fatty livers in long term (100 days) experiments with young female rats. In short term experiments (17 days), in contrast, the Toronto group[100] found no protective effect when their diet contained little methionine and choline and they[101] were unable to demonstrate a lipotropic effect of vitamin B_{12} in rats fed on diets containing low levels of betaine. Some protection against fatty infiltration was observed in short term experiments, however, when the methionine and choline levels in the diets were higher.[45,88,100]

There are some anomalies among the results obtained in studies on the lipotropic effects of vitamin B_{12} and betaine that are difficult to explain with our present knowledge. It has been generally assumed, as has been mentioned, that methionine is the principal methyl donor in the body[12,30] and that other methyl groups, such as those of betaine must pass through methionine. It has also been suggested that methyl groups synthesized in the body are incorporated into methionine before being passed to other compounds.[102] The marked lipotropic effect of vitamin B_{12} observed by Bennett and associates,[99] in contrast to the much less significant or even lack of effect observed by others.[45,88,100,101] may thus be explained by the fact that only Bennett *et al.*[99] added homocystine, an essential methionine precursor, to the methionine-deficient diet used.

Less easy to explain, however, are the observations that betaine will give rise to choline in the absence of methionine and homocystine[101] and is nearly as effective on a molar basis as choline in preventing fatty livers.[101,103] It is of course conceivable that the regeneration of methionine by a cyclic mechanism[12] could account for this result, but since methionine was already limiting in the diets used in most of these experiments and since betaine has been found to be more effective than methionine in preventing fatty livers caused by choline deficiency[104,105] the possibility of some other route from betaine to choline must be considered. The evidence presented by Stekol,[84] which suggests that betaine may be a precursor of the first two methyl groups of choline by a pathway not involving methionine, could account for these observations.

Again it is well to emphasize that such lipotropic effects as may be exerted by vitamin B_{12} are brought about through its role in the synthesis of methyl groups by the tissues and depend upon the incorporation of these methyl groups into precursors to give rise to choline.

OTHER DIETARY FACTORS

Two other dietary components which affect liver fat deposition deserve some mention. One of these, inositol, was shown to be a lipotropic factor.[106] Its role was thoroughly investigated and its effect was found to be limited to fatty livers produced in animals fed on fat-free diets.[107] The other is the nature of the dietary fat, the effect of which was originally noted by Channon and associates[108] and has since been observed by others.[109,110] An accumulation of fat in the livers of rats fed on diets deficient in essential fatty acids has also been observed.[111] There appear to be two distinct effects of fat: (1) that of the essential fatty acids, deficiencies of which cause increased liver fat deposition in the presence of choline and, (2) effects of different types of fatty acids on the severity of the fatty infiltration induced by deficiencies of lipotropic factors; the saturated fatty acids tending to increase the severity of the infiltration and the requirement for choline and the unsaturated fatty acids tending to alleviate it and lower the choline requirement.[110]

ON THE MECHANISM OF ACTION OF LIPOTROPIC FACTORS

Certain of the possible mechanisms of action of the lipotropic factors are discussed in other papers in this symposium. Nevertheless, it

seems logical to conclude with some mention of this aspect of the subject. It is evident that the factors involved in the metabolism of methyl groups exert their lipotropic effects ultimately through their contributions to choline synthesis. The recognition of choline as an essential component of phospholipids early suggested that the regulation of liver fat deposition was intimately connected with phospholipid metabolism.[6,12,15] Also, inositol and essential fatty acids, two of the other nutritional factors which influence liver fat deposition are known to be components of phospholipids. Among the lipotropic factors then, only amino acids other than methionine cannot be linked directly to the phospholipids. Therefore an understanding of the mechanisms by which nutritional factors affect liver fat deposition would appear to depend upon a knowledge of the function of phospholipids and of the sequence of occurrence of the metabolic defects caused by choline and amino acid deficiencies.

The possibility that fatty livers resulting from choline deficiency are caused by a failure in fat-transport mechanisms has received considerable attention. The evidence of a number of workers that choline stimulates phospholipid turnover and the transfer of fat from the liver to the fat depots has recently been discussed by several workers.[15,112,113] Experiments on hepatectomized dogs by Chaikoff and associates[114] suggest that the liver is the main source of phospholipids in the plasma and a progressive fall in total blood lipids and phospholipids is observed when choline is omitted from the diet.[15] The evidence using labeled fatty acids, that most of the absorbed lipid is in forms other than phopholipid,[115] makes it unlikely that fat is transported in the form of phopholipids. However, phospholipids are an integral part of the lipoprotein complex with which most of the plasma lipids are associated,[15,112] therefore, it is still possible that one of the functions of choline in protecting against fatty livers is to facilitate the transport of fat from the liver. Zilversmit[113] has discussed in detail much of the pertinent literature on this subject.

Artom[116] discusses in detail the effect of choline, presumably as part of a choline-containing complex in the mitochondria, in stimulating fatty acid oxidation. His evidence suggests that facilitation of fat oxidation is another mechanism through which choline may influence the level of liver fat.

Evidence has also been obtained of other metabolic defects in fatty livers induced by a deficiency of choline. Dianzani[117,118] has shown that these livers are characterized by a low pyridine-nucleotide content and a decreased concentration of adenosine triphosphate. He also reported that there was an increase in the proportion of reduced to oxidized pyridine nucleotides in the fatty livers, a condition that would favor fat synthesis. Study of the sequence of occurrence of these changes in relation to the development of fatty livers indicates that reductions in these important cofactors of the fatty acid cycle precede the development of fatty livers. The possibility that these changes may result in increased fat synthesis or decreased fat oxidation in the liver is a promising field for further investigation.

There is evidence, then, to suggest that in the choline-deficient rat the rate of transport of fat from the liver and the rate of oxidation of fat in the liver may both be depressed and the rate of fat synthesis may be stimulated. It is thus possible that the main question to be resolved is the relative significance of each of these defects.

The chain of events which leads to fatty infiltration of the liver in rats fed on diets deficient in protein or amino acids has not been studied extensively. It has been established experimentally that the activities of a variety of enzymes are affected to different degrees by amino acid and protein deficiencies[119] and it is obvious that the synthesis of proteins generally, including enzymes and hormones, is controlled by the supply of amino acids available. Therefore any metabolic function including phospholipid synthesis, fatty acid oxidation, or coenzyme synthesis could be affected. It has been demonstrated that the threonine-deficient diets which induce fatty livers also result in decreased phospholipid turnover,[120] reduced endogenous oxidation[121] and decreased levels of pyridine nucleotides.[122] Artom[116] has mentioned some evidence which indicates that the

rate of oxidation of fatty acids in the livers of rats fed on low-protein diets is depressed. Some evidence was obtained that the reduced endogenous oxidation and lower pyridine nucleotide levels could be detected before fatty infiltration was appreciable. This is compatible with the idea that fatty acid synthesis in the liver is favored by certain amino acid deficiencies. However, much more information is required before the true significance of these observations can be appraised. It is tempting to suggest[70,120] that amino acid deficiencies may interfere with phospholipid synthesis, a defect which would provide a link between the effects of choline and amino acid deficiencies. The histologic differentiation of the fatty livers induced by the two different types of deficiencies, however, suggests that the mechanisms may be different.

SUMMARY

Dietary deficiencies of choline, essential amino acids, essential fatty acids and inositol can under certain conditions give rise to an accumulation of liver fat. A dietary deficiency of choline can be very largely, if not completely, alleviated by providing in the diet sources of labile methyl groups, such as betaine or methionine, which permit choline synthesis in the body. Also, choline deficiency is made less severe if folic acid and vitamin B_{12}, which are involved in the synthesis of ethanolamine and methyl groups, are included in the diet. The fatty liver induced by the feeding of low-protein diets which contain choline appears in some cases to be the result of a specific amino acid deficiency which may be induced by an amino acid imbalance. It appears to be influenced as well, however, by the over-all level of dietary protein and by the levels of certain dispensible amino acids, relationships which require further study. Although many of the nutritional interrelationships that influence the production of fatty livers have been worked out, and although a number of changes in the rates of metabolic reactions and in the levels of metabolically important compounds have been observed in the livers of rats fed on diets deficient in choline or essential amino acids, an integrated picture of the mechanisms responsible for the production of fatty livers has not, as yet, been obtained.

REFERENCES

1. Griffith, W. H.: Choline; in *The Biological Action of the Vitamins* (ed. by E. A. Evans). Univ. of Chicago Press, Chicago, 1942, p. 169.
2. Best, C. H. and Lucas, C. C.: *Vitamins and Hormones* 1: 1, 1943.
3. McHenry, E. W. and Patterson, J. M.: *Physiol. Rev.* 24: 128, 1944.
4. Peters, J. P. and Van Slyke, D. D.: The lipids; in *Quantitative Clinical Chemistry*, ed. 2, Vol. 1. Williams and Wilkins, Baltimore, 1946, pp. 423–438.
5. Best, C. H.: *Fed. Proc.* 9: 506, 1950.
6. Best, C. H. and Lucas, C. C.: Choline malnutrition; in *Clinical Nutrition* (ed. by N. Jolliffe, F. F. Tisdale and P. B. Cannon). Hoeber, New York, 1950, pp. 561–585.
7. Zucker, T. F. and Zucker, L. M.: *Vitamins and Hormones* 8: 1, 1950.
8. Woods, R.: *Borden's Rev. Nutr. Res.* 12: 11, 23, 1951.
9. Jukes, T. H. and Stokstad, E. L. R.: *Vitamins and Hormones* 9: 1, 1951.
10. Du Vigneaud, V.: *A Trail of Research in Sulfur Chemistry and Metabolism and Related Fields.* Cornell Univ. Press, Ithaca, 1952.
11. Best, C. H.: *Nutr. Rev.* 11: 321, 1953.
12. Griffith, W. H. and Nyc, J. F.: Choline; in *The Vitamins* (ed. by W. H. Sebrell and R. S. Harris). Academic Press, New York, 1954, pp. 2–104.
13. Best, C. H., Lucas, C. C., and Ridout, J. H.: *Ann. New York Acad. Sc.* 57: 646, 1954.
14. Best, C. H., Lucas, C. C., and Ridout, J. H.: *Brit. M. Bull.* 12: 9, 1956.
15. Best, C. H.: *Proc. Roy. Soc., London. S. B.* 145: 151, 1956.
16. Macleod, J. J. R.: *Physiol. Rev.* 4: 21, 1924.
17. Bliss, S. W.: *J. Metab. Res.* 2: 385, 1922.
18. Allan, F. N., Bowie, D. J., Macleod, J. J. R., and Robinson, W. L.: *Brit. J. Exper. Path.* 5: 75, 1924.
19. Fisher, N. F.: *Am. J. Physiol.* 67: 634, 1924.
20. Hershey, J. M.: *Am. J. Physiol.* 93: 657P, 1930.
21. Hershey, J. M. and Soskin, S.: *Am. J. Physiol.* 98: 74, 1931.
22. Best, C. H., Hershey, J. M., and Huntsman, M. E.: *J. Physiol.* 75: 56, 1932.
23. Best, C. H., Hershey, J. M., and Huntsman, M. E.: *Am. J. Physiol.* 101: 7p, 1932.
24. Best, C. H. and Huntsman, M. E.: *J. Physiol.* 75: 405, 1932.
25. Best, C. H. and Huntsman, M. E.: *J. Physiol.* 83: 255, 1935.

26. CHANNON, H. J. and WILKINSON, H.: *Biochem. J.* 29: 350, 1935.
27. BEST, C. H. and CHANNON, H. J.: *Biochem. J.* 29: 2651, 1935.
28. BEST, C. H., GRANT, R., and RIDOUT, J. H.: *J. Physiol.* 86: 337, 1936.
29. CHANNON, H. J., LOACH, J. V., LOIZIDES, P. A., MANIFOLD, M. C., and SOLIMAN, G.: *Biochem. J.* 32: 976, 1938.
30. BEESTON, A. W., CHANNON, H. J., LOACH, J. V., and WILKINSON, H.: *Biochem. J.* 30: 1040, 1936.
31. BEESTON, A. W. and CHANNON, H. J.: *Biochem. J.* 30: 280, 1936.
32. TUCKER, H. F. and ECKSTEIN, H. C.: *J. Biol. Chem.* 121: 479, 1937.
33. CHANNON, H. J., MANIFOLD, M. C., and PLATT, A. P.: *Biochem. J.* 32: 969, 1938.
34. BEST, C. H. and RIDOUT, J. H.: *J. Physiol.* 97: 489, 1940.
35. SINGAL, S. A. and ECKSTEIN, H. C.: *Proc. Soc. Exper. Biol. & Med.* 41: 512, 1939.
36. TUCKER, H. F. and ECKSTEIN, H. C.: *J. Biol. Chem.* 126: 117, 1938.
37. CHANNON, H. J., MANIFOLD, M. C., and PLATT, A. P.: *Biochem. J.* 34: 866, 1940.
38. CHANNON, H. J., MILLS, C. T., and PLATT, A. P.: *Biochem. J.* 37: 483, 1943.
39. TUCKER, H. F., TREADWELL, C. R., and ECKSTEIN, H. C.: *J. Biol. Chem.* 135: 85, 1940.
40. TREADWELL, C. R., GROOTHIUS, M. G., and ECKSTEIN, H. C.: *J. Biol. Chem.* 142: 653, 1942.
41. HORNING, M. G. and ECKSTEIN, H. C.: *J. Biol. Chem.* 155: 49, 1944.
42. TREADWELL, C. R., TIDWELL, H. C., and GAST, J. H.: *J. Biol. Chem.* 156: 237, 1944.
43. BEVERIDGE, J. M. R., LUCAS, C. C., and O'GRADY, M. K.: *J. Biol. Chem.* 154: 9, 1944.
44. BEVERIDGE, J. M. R., LUCAS, C. C., and O'GRADY, M. K.: *J. Biol. Chem.* 160: 505, 1945.
45. GRIFFITH, W. H. and MULFORD, D. J.: *J. Nutrition* 21: 633, 1941.
46. MULFORD, D. J. and GRIFFITH, W. H.: *J. Nutrition* 23: 91, 1942.
47. KREHL, W. A., HENDERSON, L. M., DE LA HUERGA, J., and ELVEHJEM, C. A.: *J. Biol. Chem.* 166: 531, 1946.
48. SINGAL, S. A., SYDENSTRICKER, V. P., and LITTLEJOHN, J. M.: *J. Biol. Chem.* 176: 1063, 1948.
49. HAWK, E. A. and ELVEHJEM, C. A.: *J. Nutrition* 49: 495, 1953.
50. LITWACK, G., HANKES, L. V., and ELVEHJEM, C. A.: *Proc. Soc. Exper. Biol. & Med.* 81: 441, 1952.
51. SINGAL, S. A., HAZAN, S. J., SYDENSTRICKER, V. P., and LITTLEJOHN, J. M.: *J. Biol. Chem.* 200: 867, 1953.
52. HARPER, A. E., MONSON, W. J., BENTON, D. A., and ELVEHJEM, C. A.: *J. Nutrition* 50: 383, 1953.
53. HARPER, A. E., MONSON, W. J., ARATA, D. A., BENTON, D. A., and ELVEHJEM, C. A.: *J. Nutrition* 51: 523, 1953.
54. WINJE, M. E., HARPER, A. E., BENTON, D. A., BOLDT, R. E., and ELVEHJEM, C. A.: *J. Nutrition* 54: 155, 1954.
55. HARPER, A. E., WINJE, M. E., BENTON, D. A., and ELVEHJEM, C. A.: *J. Nutrition* 56: 187, 1955.
56. DESHPANDE, P. D., HARPER, A. E., QUIROS-PEREZ, F., and ELVEHJEM, C. A.: *J. Nutrition* 57: 415, 1955.
57. SAUBERLICH, H. E.: *Fed. Proc.* 12: 263, 1953.
58. HARPER, A. E., BENTON, D. A., WINJE, M. E., MONSON, W. J., and ELVEHJEM, C. A.: *J. Biol. Chem.* 209: 165, 1954.
59. SHILS, M. E. and STEWART, W. B.: *Proc. Soc. Exper. Biol. & Med.* 85: 298, 1954.
60. SHILS, M. E., FRIEDLAND, I., and STEWART, W. B.: *Proc. Soc. Exper. Biol. & Med.* 87: 473, 1954.
61. SHILS, M. E. and STEWART, W. B.: *Proc. Soc. Exper. Biol. & Med.* 87: 629, 1954.
62. COLE, A. S. and SCOTT, P. P.: *Brit. J. Nutrition* 8: 125, 1954.
63. BEST, C. H., HARTROFT, W. S., LUCAS, C. C., and RIDOUT, J. H.: *Brit. M. J.* 1: 1439, 1955.
64. DESHPANDE, P. D., HARPER, A. E., and ELVEHJEM, C. A.: *J. Nutrition* 62: 503, 1957.
65. DESHPANDE, P. D., HARPER, A. E., and ELVEHJEM, C. A.: *J. Biol. Chem.* 230: 327, 1958.
66. ECKSTEIN, H. C.: *J. Biol. Chem.* 195: 167, 1952.
67. GRIFFITH, W. H. and WADE, N. J.: *J. Biol. Chem.* 132: 627, 1940.
68. HARPER, A. E., MONSON, W. J., BENTON, D. A., WINJE, M. E., and ELVEHJEM, C. A.: *J. Biol. Chem.* 206: 151, 1954.
69. HARPER, A. E., BENTON, D. A., WINJE, M. E., and ELVEHJEM, C. A.: *J. Biol. Chem.* 209: 171, 1954.
70. LUCAS, C. C. and RIDOUT, J. H.: *Canad. J. Biochem. & Physiol.* 33: 25, 1955.
71. NINO-HERRERA, H., HARPER, A. E., and ELVEHJEM, C. A.: *J. Nutrition* 53: 469, 1954.
72. HARPER, A. E., BENTON, D. A., WINJE, M. E., and ELVEHJEM, C. A.: *J. Biol. Chem.* 209: 159, 1954.
73. BENTON, D. A., HARPER, A. E., WINJE, M. E., and ELVEHJEM, C. A.: *J. Biol. Chem.* 214: 677, 1955.
74. WOMACK, M., KEMMERER, K. S., and ROSE, W. C.: *J. Biol. Chem.* 121: 403, 1937.
75. DU VIGNEAUD, V., CHANDLER, J. P., MOYER, A. W., and KEPPEL, D. M.: *J. Biol. Chem.* 131: 57, 1939.
76. DU VIGNEAUD, V., CHANDLER, J. P., COHN, M., and BROWN, C.: *J. Biol. Chem.* 134: 787, 1940.
77. STETTEN, DE W.: *J. Biol. Chem.* 138: 437, 1941.
78. DU VIGNEAUD, V., COHN, M., CHANDLER, J. P.,

SCHENK, J. R., and SIMMONDS, S.: *J. Biol. Chem.* 140:625, 1941.
79. CHANDLER, J. P. and DU VIGNEAUD, V.: *J. Biol. Chem.* 135:223, 1940.
80. CHALLENGER, F.: *Quart. Rev. Chem. Soc.* 9:255, 1955.
81. BORSOOK, H. and DUBNOFF, J. W.: *J. Biol. Chem.* 169:247, 1947.
82. DU VIGNEAUD, V., SIMMONDS, S., CHANDLER, J. P., and COHN, M.: *J. Biol. Chem.* 165:639, 1946.
83. CANTONI, G. L.: *J. Am. Chem. Soc.* 74:2942, 1952.
84. STEKOL, J. A.: AM. J. CLIN. NUTRITION 6:200, 1958.
85. WELCH, A. D.: *Proc. Soc. Exper. Biol. & Med.* 35:107, 1936.
86. CHANNON, H. J. and SMITH, J. A. B.: *Biochem. J.* 30:115, 1936.
87. WELLS, I.: AM. J. CLIN. NUTRITION 6:254, 1958.
88. STRENGTH, D. R., SCHAEFFER, A. E., and SALMON, W. D.: *J. Nutrition* 45:329, 1951.
89. TOENNIES, G., BENNETT, M. A., and MEDES, G.: *Growth* 7:251, 1943.
90. BENNETT, M. A., MEDES, G., and TOENNIES, G.: *Growth* 8:59, 1944.
91. MEDES, G., FLOYD, N. F., and CAMMAROTI, M. S.: *Growth* 8:89, 1944.
92. DU VIGNEAUD, V., SIMMONDS, S., CHANDLER, J. P., and COHN, M.: *J. Biol. Chem.* 159:755, 1945.
93. DU VIGNEAUD, V., RESSLER, C., and RACHELE, J. R.: *Science* 112:267, 1950.
94. BENNETT, M. A.: *J. Biol. Chem.* 187:751, 1950.
95. STEKOL, J. A. and WEISS, K.: *J. Biol. Chem.* 186:343, 1950.
96. GILLIS, M. B. and NORRIS, L. C.: *J. Biol. Chem.* 179:487, 1949.
97. JUKES, T. H. and STOKSTAD, E. L. R.: *J. Nutrition* 43:459, 1951.
98. KAMIN, H. and HANDLER, P.: *Ann. Rev. Biochem.* 26:442, 1957.
99. BENNETT, M. A., JORALEMON, J., and HALPERN, P. E.: *J. Biol. Chem.* 193:285, 1951.
100. BEST, C. H., LUCAS, C. C., PATTERSON, J. M., and RIDOUT, J. H.: *Canad. J. M. Sc.* 31:135, 1953.
101. YOUNG, R. J., LUCAS, C. C., PATTERSON, J. M., and BEST, C. H.: *J. Biol. Chem.* 224:341, 1957.
102. STEKOL, J. A., WEISS, S., SMITH, P., and WEISS, K.: *J. Biol. Chem.* 201:299, 1953.
103. BEST, C. H., LUCAS, C. C., RIDOUT, J. H., and PATTERSON, J. M.: *J. Biol. Chem.* 186:317, 1950.
104. HARPER, A. E. and BENTON, D. A.: *Biochem. J.* 62:440, 1956.
105. YOUNG, R. J., LUCAS, C. C., PATTERSON, J. M., and BEST, C. H.: *Canad. J. Biochem. & Physiol.* 34:713, 1956.
106. GAVIN, G. and MCHENRY, E. W.: *J. Biol. Chem.* 141:619, 1941.
107. BEST, C. H., RIDOUT, J. H., PATTERSON, J. M., and LUCAS, C. C.: *Biochem. J.* 48:448, 1951.
108. CHANNON, H. J. and WILKINSON, H.: *Biochem. J.* 30:1033, 1936.
109. HARTROFT, W. S.: *Fed. Proc.* 14:655, 1955.
110. BENTON, D. A., SPIVEY, H. E., QUIROS-PEREZ, F., HARPER, A. E., and ELVEHJEM, C. A.: *Proc. Soc. Exper. Biol. & Med.* 94:100, 1957.
111. ENGEL, R. W.: *J. Nutrition* 24:175, 1942.
112. BEVERIDGE, J. M. R.: *Canad. J. Biochem. & Physiol.* 34:361, 1956.
113. ZILVERSMIT, D. B. and DILUZIO, N. R.: AM. J. CLIN. NUTRITION 6:235, 1958.
114. FISHLER, M. C., ENTENMAN, C., MONTGOMERY, M. L., and CHAIKOFF, I. L.: *J. Biol. Chem.* 150:47, 1943.
115. BLOOM, B., CHAIKOFF, I. L., REINHARDT, W. O., and DAUBEN, W. G.: *J. Biol. Chem.* 189:261, 1951.
116. ARTOM, C.: AM. J. CLIN. NUTRITION 6:221, 1958.
117. DIANZANI, M. U.: *Biochem. et Biophys. Acta* 17:391, 1955.
118. DIANZANI, M. U.: *Biochem. J.* 65:116, 1957.
119. KNOX, W. E., AUERBACH, V. H., and LIN, E. C. C.: *Physiol. Rev.* 36:164, 1956.
120. SINGAL, S. A., HAZAN, S. J., SYDENSTRICKER, V. P., and LITTLEJOHN, J. M.: *J. Biol. Chem.* 200:875, 1953.
121. ARATA, D. A., HARPER, A. E., SVENNEBY, G., WILLIAMS, J. N., JR., and ELVEHJEM, C. A.: *Proc. Soc. Exper. Biol. & Med.* 87:544, 1954.
122. ARATA, D. A., SVENNEBY, G., WILLIAMS, J. N., JR., and ELVEHJEM, C. A.: *J. Biol. Chem.* 219:327, 1956.

DISCUSSION

Dr. A. E. Axelrod (University of Pittsburgh, Pittsburgh, Pennsylvania): Dr. Harper has summarized a very complicated field, and there is not much left for me to do. I would like, however, to mention two old observations which have not been clarified. The first is that the feeding of biotin produces a fatty liver. Is it an effect purely of growth that you get with biotin or is there something further that we need to think about with this factor? The other is the "hormone" lipocaic. Is the effect of this factor purely one of choline? Are there any proteolytic enzymes involved? Is this the entire story? Is there anything further we need to say in this field?

I would like to make a few comments relative to the mechanism of action of these various factors. Obviously, we know very few concrete facts. I am very gratified that the biochemists are beginning to approach this problem now by looking for the actual enzyme systems which may be involved. They were mentioned many times today. I would like to throw out the

concept that perhaps we should look a little closer at the structure of the particles of the cell which may be involved, particularly to the mitochondria as has been already suggested today. Is it possible that the structure of the mitochondria is markedly changed in the absence of some of these factors and, as a result of these changes, we see the enzymatic changes that produce these fatty livers?

Finally, we have not said anything about the role of these factors and their interrelationship with various hormones. I know of only one case—perhaps members of the audience may know others—where hormones may be activated or inactivated in these particular nutritional states. I would like to see this matter discussed further by the group.

Dr. Hartroft: I would like to thank Dr. Harper for a very clear and lucid exposition of many interrelations in this area which have confused the morphologist. I would like also to underline and emphasize, if I may, his statement that various types of fatty liver affect quite different portions of the hepatic lobule. Dr. Harper mentioned that the chief site of deposition of fat in choline-deficient rats is centrolobular, and that in states of tryptophan, lysine, and threonine deficiencies the abnormal fat is periportal in position; facts which he himself has reported and we have certainly confirmed. These localizations are very definite in the rat. Any pathologist can take a series of these sections, completely jumbled, and with perfect confidence pick out the livers of choline-deficient animals from those of threonine-deficient animals, or from rats fed a 3 per cent casein, choline-supplemented diet.

Consequently, to emphasize that choline-deficiency may produce an abnormal increase in liver fat up to 15 or 20 per cent of its wet weight, and that threonine, lysine, and tryptophan-deficiency does this only up to 10 per cent of wet weight is misleading, in my opinion. If the cells involved in each condition are considered, those in the centrolobular portions of the choline-deficient fatty liver are loaded with fat, it is true, but no more so than cells in the periportal regions in the amino acid-deficient type of fatty liver. The cells so affected may be less numerous in the livers of the threonine, tryptophan, and lysine-deficient types, but individually they are just as severely affected.

There are other features which enable us to separate these types of fatty livers on a cytologic level. In the choline-deficient fatty liver, the individual cell contains droplets of fat in its cytoplasm which later fuse into large globules that displace the hepatic nucleus to one side of the cell in a signet ring fashion. In the type of fatty liver studied so well at the Wisconsin laboratories, this same cytologic change may occur, but more frequently the fat accumulates inside little intracytoplasmic compartments which persist and do not fuse together, so the nucleus does not become displaced. Examination of a single cell, in favorable instances, may therefore suggest to the observer which type of fatty liver one is dealing with, even without knowing the history of the experiment.

Further advances in this field on the enzymologic and cytologic level will perhaps not come from studies of liver homogenates, but from the laboratories of the microchemist and the microenzymologist who study only small groups of cells, or even single cells.

Dr. Stekol: To pick up Dr. Axelrod's point on lipocaic, this was resurrected by Leites in a paper published in Russian, unfortunately, which has been translated and distributed. They found that the lipotropic effect of choline and methionine is abolished in animals in which the portion of the pancreas which produces lipocaic is removed from circulation. Only upon administration of lipocaic together with choline and methionine do they find a lipotropic effect. The conclusion was that lipocaic, whatever it happens to be and, by the way, they purified it and it is different from the Dragstedt preparation, has some sort of activating effect on choline, almost an enzymatic effect, in producing the lipotropic effect.

Incidentally, in Russia this lipocaic is manufactured on a large scale and it is distributed to hospitals for treatment of several liver diseases in humans. I have the translation if you want it. It is a long one.

Dr. Wilgram: In addition to Dr. Harper's comment concerning the distribution of fat in different parts of the liver lobule in rats, I would like to state that we have recently studied monkeys in choline deficiency and, to our great surprise (I believe, Dr. Hartroft, I sent you some sections) the fat in choline-deficient monkeys accumulates not centrally as in rats but in the periportal area. So not only have you got in the same species different sites of fat accumulation in different deficiency states, but for some reason unknown to me different species handle the same deficiency in a different way.

Dr. Hartroft: That is why I was careful to say "rat." I had seen these sections, and I was hoping Dr. Wilgram would mention them, but I did not feel free to. In the sections he sent me the fat was very definitely periportal.

Dr. Cornatzer: We have investigated the lecithin and cephalin content of liver mitochondria and nuclei in choline deficient rats maintained on a 5 per cent casein diet supplemented with 1 per cent guanidoacetic acid for 2.5 weeks. A decrease occurred in the lecithin phosphorus of the mitochondria when a choline deficiency was produced. The administration of a single dose of choline (40, 75 or 150 mg) six hours before sacrificing the animals maintained on a 5 per cent casein diet supplemented with 1 per cent guanidoacetic for 2.5 weeks produced a significant increase in lecithin content of the mitochondria. The effect of a single dose of choline (150 mg) was demonstrated in the liver mitochondria within 3, 6 or 10 hours following administration (*Am. J. Med.* 21: 136, 1956).

Dr. Harper: Fortunately, members of the audience have already answered some of the questions. Re-

garding the point made by Dr. Axelrod about "biotin" fatty livers, this type of phenomenon has also been observed with deficiencies of some other B-vitamins. As far as I know, it is brought about by this procedure: The animals are reared on a diet deficient in one of the B-vitamins. Then they are given a diet containing an adequate level of the vitamin previously omitted from the diet. The observation is that within a short time after the animals have consumed this diet, fat accumulates in the liver. As far as I know, if the animals are left on this diet for a period of time, a matter of a week or ten days, the liver fat levels return to normal. I have heard it interpreted by some of the people who worked on this problem at Wisconsin that this is a case in which the animal has taken in a tremendous quantity of calories after being on a relatively low caloric intake; then the liver becomes overloaded with carbohydrate or fat which leads to the accumulation of fat in the liver. This is perfectly normally removed as long as the animal is continued on a diet containing the vitamin.

With regard to lipocaic, Dr. Stekol's comments are adequate for that. I know of very little other recent work. I think everyone is familiar with the suggestion that lipocaic provides methionine or pancreatic enzymes.

With regard to the cellular structure, there are many people here more competent than I am to discuss the relationship of phospholipids to cellular structure or the relation of cellular structure to function, but I think there is no doubt that this must be kept in mind in all of these studies to determine the mechanism of fatty livers.

I was glad to hear Dr. Hartroft's comment. Of course, he has done a great deal of the histologic work on both choline and protein deficiencies. I must admit that I am not much of a histologist, but when the histologic work was being done in our laboratory, in some instances with animals on low protein diets I found it very hard to believe that the slide under the microscope corresponded to the animal on which the fat determination had been made, because, as he says, even with relatively low levels of fat, as determined chemically, quite wide areas of the cells can be completely loaded with fat. In the laboratory we describe it as a network appearance with areas containing normal cells interspersed among areas in which the cells are loaded with fat. This is a very important point to keep in mind in many of our biochemical studies. Certainly with many of the low-protein diets I suspect there may be as much as 50 per cent of the liver normal in appearance while 50 per cent may be loaded with fat. We are certainly not getting a complete picture by working with homogenates.

I was quite interested to hear Dr. Wilgram's comment. I have nothing more to say on that except that I would like to ask what the protein level was in the choline-deficient diet the monkeys were fed. We have observed in some animals which we had on a choline-deficient, low protein diet that we got varying degrees of fatty infiltration.

Dr. Wilgram: The protein content of this diet was about 17 per cent, and this was sufficient judging by the monkeys' growth. Despite being deficient, they grew. One female monkey even became pregnant in the laboratory.

Dr. Harper: Certainly 17 per cent of protein should be adequate. With regard to the last point, about the decrease in lecithin in choline-deficient animals and the rapid synthesis of lecithin after the injection of choline. I believe there must be some relationship between this and the mechanism by which fatty livers are induced.

Role of Choline and Methionine Antagonists in Metabolism

IBERT C. WELLS, PH.D.*

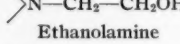
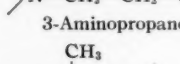
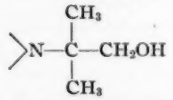
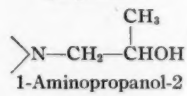
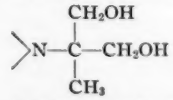
WE HAVE described previously[1] the study *in vitro* of a series of analogues and homologues of choline as substrates for the enzyme choline dehydrogenase. These compounds are shown in Table I. Of these compounds, only the quaternary bases, with the exception of triethylcholine and triethylhomocholine, were oxidized appreciably by the enzyme. However, the enzyme exhibited some affinity for all the remaining compounds in that they could depress competitively the oxidation rate of choline.

Two of the most potent of these inhibitors, 2-amino-2-methylpropanol-1 and α,α-dimethyltriethylcholine were selected for further study. It was found[1] that *in vitro* these compounds inhibited the synthesis of methionine from choline but not from betaine. This inhibition was attributed to the ability of these compounds to depress the rate of choline oxidation by choline dehydrogenase.[2–4] *In vivo*, however, these compounds were not capable[5] of significantly reducing the rate of choline oxidation. This conclusion was drawn from their inability to inhibit the growth of young, male rats existing on a methionine-deficient diet supplemented with equivalent amounts of choline and DL-homocystine. Another antimetabolic role of these inhibitors was apparent from the observation that when they were added to diets containing adequate amounts of methionine but no choline, hemorrhagic kidney degeneration and death of the animals resulted. The diets without the inhibitors produced only a low incidence of hemorrhagic kidney degeneration, but all the animals had fatty livers. The effect of these compounds was prevented by supplements of choline.[5]

The basic diet employed for the remainder of these studies is similar to that used by Griffith[6] and is given in Table II. A plot of various dietary concentrations of the inhibitors against the dietary concentration of choline necessary to prevent hemorrhagic kidney degeneration is shown in Figure 1. A concentration of 100 mg/100 g of choline chloride was sufficient to offset the effect of 0.33 per cent 2-amino-2-methylpropanol. Concentrations of 0.67 and

TABLE I

Analogues and Homologues of Choline as Substrates for the Enzyme Choline Dehydrogenase

Parent amine	Some derivatives studied
 Ethanolamine	Choline, dimethylethylcholine, diethylmethylcholine, triethylcholine
 3-Aminopropanol-1	Homocholine, triethylhomocholine
 2-Amino-2-methylpropanol-1	α,α-Dimethylcholine α,α-Dimethyltriethylcholine
 1-Aminopropanol-2	β-Methylcholine β-Methyltriethylcholine
 2-Amino-2-methylpropanediol-1,3	α-Methyl-α-hydroxymethylcholine α-Methyl-α-hydroxymethyltriethylcholine

From the Dept. of Biochemistry, State University of New York, Medical College at Syracuse, New York.

* Associate Professor in Biochemistry, State University of New York Medical Center, Syracuse.

Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc., New York, New York.

1.0 per cent of the inhibitor required 150 and 200 mg/100 g of choline chloride, respectively. These amounts of choline not only protected the animals from hemorrhagic kidney degeneration, but also from fatty infiltration of the liver. Since hemorrhagic kidney degeneration appeared occasionally during several repetitions of the experiment in which the combination of 0.33 per cent 2-amino-2-methylpropanol-1 and 100 mg/100 g choline chloride was administered, this concentration of choline is reasonably close to the actual concentration of choline required.

The results of the study of α,α-dimethyltriethylcholine and choline were less certain. A level of 0.8 per cent of this inhibitor required a concentration of choline chloride of about 133 mg/100 g whereas a level of 1.6 per cent increased the choline requirement to about 200 mg/100 g. The concentration of choline required to counteract the effect of 2.4 per cent α,α-dimethyltriethylcholine chloride was only slightly higher than that required to reverse the effect of 1.6 per cent of the inhibitor. Unlike the findings with 2-amino-2-methylpropanol-1, those concentrations of choline which afforded kidney protection to most of the animals receiving each level of concentration of α,α-dimethyltriethylcholine were insufficient to reduce the total liver lipid concentrations to the control values. However, higher concentrations of choline were effective in this respect. Extrapolation of the curves in Figure 1 to zero inhibitor concentration gives a value of about 50 mg/100 g which would be the choline concentration normally required to prevent hemorrhagic kidney degeneration. This concentration of choline corresponds to a daily intake of about 3 mg of choline, a value in the same range as that determined by Griffith.[6]

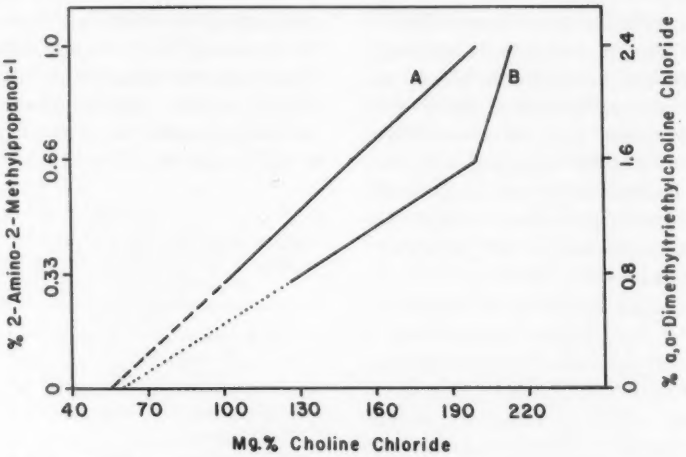


Fig. 1. The dietary concentration of choline chloride necessary to prevent hemorrhagic kidney degeneration caused by feeding 2-amino-2-methylpropanol-1 and α,α-dimethyltriethylcholine chloride at various concentrations in the diet. Curve A, 2-amino-2-methyl-propanol-1; curve B, α,α-dimethyltriethylcholine chloride.

TABLE II

Basic Diet Employed

	%	
Casein (GBI—vitamin test)	18	
Crisco	15	
Wesson oil	2	
Cod liver oil	4	
Sucrose	17	
Glucose	40	
Salt mixture (Phillips and Hart)	4	
Thiamin hydrochloride	0.3	mg/100 g
Riboflavin	0.4	
Pyridoxine hydrochloride	0.3	
Calcium pantothenate	1.0	
Nicotinic acid	1.0	

On the basis of preventing hemorrhagic kidney degeneration, the calculated molecular inhibition index for 2-amino-2-methylpropanol-1 is about 9. The index for α,α-dimethyltriethylcholine is somewhat less than nine over the range of concentrations and acts strongly as a competitive inhibitor of choline.

In accordance with the hypothesis of Welch[7] that the intact choline molecule is necessary for the prevention of hemorrhagic kidney degeneration and fatty infiltration of the liver in rats, it was concluded that α,α-dimethyltriethylcholine is a competitive inhibitor of choline utilization in the kidney and in the liver, while 2-amino-2-methylpropanol-1 inhibits the utilization of choline mainly in the kidney and perhaps to a slight extent in the liver.

The apparent organ specificity of 2-amino-2-methylpropanol-1 has obvious implications in the therapeutic applications of antimetabolites. Not only do the systems utilizing choline in rat liver and kidney exhibit a difference in susceptibility to 2-amino-2-methylpropanol-1 and α,α-dimethyltriethylcholine, but the choline utilizing systems in *Neurospora crassa* (strain 34486) and *Saccharomyces carlsbergensis* and in the rabbit ileum are susceptible to still other choline antagonists.[8,9] A list of the effective antagonists in these systems is shown in Table III.

TABLE III

Antimetabolites of Choline

A.	In neurospora crassa (strain 34486):
	Ethanolamine
	β-Methylcholine
	α-Methyl-α-hydroxymethylcholine
B.	In saccharomyces carlsbergensis
	Methyldiethylhomocholine
	1-Diethylaminopropanol-2
	α,α-Dimethylcholine
C.	In rabbit ileum
	Triethylcholine
	β-Methyltriethylcholine
	α-Methyl-α-hydroxymethylcholine

Recently we have been studying the abilities of labile-methyl compounds other than choline to overcome the effects of 2-amino-2-methylpropanol-1[10] and in trying to determine the mechanism of action of this inhibitor. The labile-methyl compounds studied are DL-methionine, betaine and β-dimethylpropiothetin. Normally 1 mg of choline chloride is replaced in the diet of the rat by 4 mg of DL-methionine or betaine hydrochloride[11] and theoretically by about 4 mg of β-dimethylpropiothetin hydrochloride. Since 100 mg/100 g (0.72 mMol/100 g) choline chloride in the diet effectively prevents the production of hemorrhagic kidney degeneration by 2-amino-2-methylpropanol-1 at a dietary concentration of 0.33 per cent (3.7 mMol per cent) it might be expected that 400 mg/100 g (2.7 mMol/100 g) of DL-methionine and equal molecular amounts of betaine hydrochloride and β-dimethylpropiothetin hydrochloride would also offset the effects of the same concentration of the inhibitor. As seen in Table IV, the amounts of methionine, betaine, and dimethylpropiothetin required were much higher than was expected. In the presence of the inhibitor the amount of methionine equivalent to 1 mg of choline was about four and one-half that expected and the amounts of betaine and β-dimethylpropiothetin exceeded this.

TABLE IV

Dietary Concentrations of Methyl Donors Necessary to Replace 100 mg/100 g Choline Chloride in Presence of 0.33% 2-Amino-2-methylpropanol[10]*

Methyl donor	Concentration expected		Concentration found	
	mg/100 g	*mMol/100 g*	*mg/100 g*	*mMol/100 g*
DL-Methionine hydrochloride	400	2.7	1800	12.2
Betaine hydrochloride	400	2.6	>2800	>18.2
β-Dimethylpropiothetin hydrochloride	445	2.6	>2059	>12.2

* 100 mg/100 g choline chloride is equivalent to 0.72 mMol/100 g; 0.33% 2-amino-2-methylpropanol-1 is equivalent to 3.7 mMol/100 g.

Since, of the labile-methyl compounds studied, methionine is the nearest precursor of choline in its biosynthesis, it was concluded that 2-amino-2-methylpropanol-1 inhibits the transfer of the methionine methyl group in choline synthesis in the kidney as well as inter-

fering with the utilization of choline in this organ. This conclusion seemed reasonable since β-methylcholine had been found to inhibit the methylation of dimethylaminoethanol in *N. crassa*.[8] However, other possible mechanisms present themselves. First, the methionine might be methylating the inhibitor. Although dietary methionine is sufficient for this purpose, it seems unlikely since in the presence of catalytic amounts of methionine, betaine and β-dimethylpropiothetin should serve as well. In addition, if it be assumed that it is the relationship of the concentration of free choline to that of inhibitor in the kidney which is of importance in counteracting the inhibitor, then it is possible to explain the apparent inhibition of choline synthesis on a kinetic basis. The normal concentration of free choline in the kidney would be, in the absence of dietary choline, determined by the balance between the rate of synthesis and the rates of utilization and catabolism. If this normal equilibrium concentration were not sufficient to overcome the inhibitor, a higher equilibrium concentration could be established by increasing the concentration of methionine available for transmethylation. Increasing the concentration of betaine or β-dimethylpropiothetin would not be effective because the homocysteine concentration would be limiting. Experiments done thus far have not permitted an unequivocal decision concerning these two mechanisms.

Whether or not 2-amino-2-methylpropanol-1 antagonizes the transmethylation of methionine remains to be demonstrated by further experiments. However, ethionine has been shown conclusively to be an antagonist of methionine and the discussion to follow will be a survey of the work done with this compound.

ETHIONINE

Dyer[12] in 1938 reported the synthesis of ethionine together with the observation that it would not support the growth of rats on a diet devoid of cystine and containing suboptimal amounts of methionine. This was confirmed by Shen and Lewis[13] who also observed that the administered ethionine was largely excreted as extra sulfur in the urine. Harris and Kohn[14] obtained evidence in *Escherichia coli* that ethionine was a competitive inhibitor of methionine.

Ethionine has been found to be an antagonist of methionine as judged by several biologic criteria: growth, both in animals and microorganisms, protein synthesis and transmethylation.

Stekol and Weiss[15] reported that D- and L-ethionine as well as DL-ethionine were effective growth inhibitors in rats and that their effect was alleviated by either D- or L-methionine. Choline was also found to be effective in alleviating the effect of the inhibitor due probably to its ability to spare methionine.

Simpson *et al.*[16] obtained evidence which indicated that ethionine competitively inhibited the incorporation of methionine into the liver proteins of intact rats, Table V. Ethionine has a similar effect in bacteria[17] and studies of the free amino acid concentrations in plasma and tissues after ethionine administration are in agreement with this concept.[18,19] Not only does ethionine inhibit the incorporation of methionine into proteins, but much work[20–22] has indicated that ethionine itself is incorporated into proteins. These observations have led to the hypothesis that the effects of ethionine are to be attributed to abnormal protein formation.[20] Apparently ethionine is incorporated into the protein in some of the same sites that would normally be occupied by methionine. The affinity for incorporation of ethionine, however, is only $^1/_{600}$ that for methionine. Once ethionine is incorporated, it is not readily released by methionine.[21]

TABLE V

Effect of Ethionine and of Ethionine Plus Methionine on Uptake of Methionine by Liver Proteins of Rats[16]

Experiment No.	Methionine injected *mMol*	Specific activity of protein after (counts/min/μM)*		% inhibition
		Methionine	Methionine + ethionine	
2	0	67	31	53
3	0.92	213	200	6

* In all experiments, a tracer dose (6.7-13.4 μM) of methionine was given by intraperitoneal injection. Each animal received 0.92 mMol of ethionine intraperitoneally in divided doses.

In contrast to these findings, it has been reported[23] that ethionine in concentrations up to 0.005 M did not inhibit the incorporation of leucine into protein by rat liver homogenates. No inhibition of protein synthesis in the pancreas of ethionine treated rats was observed.[24]

In 1950, Farber *et al.*[25] reported an interference with lipid metabolism by ethionine. It was found that the intraperitoneal administration of ethionine to fasted female rats produced a rise in liver lipid concentration which was clearly discernible after 12 hours and thereafter increased with time (Table VI).

TABLE VI

Effect of Some Amino Acids on Fatty Liver Produced by Ethionine[25]*

No. of rats	Test substance	Dose *mMol*	Total lipid %, *wet wt*
21	None, no ethionine	—	6.1 ± 0.3
22	None, with ethionine	—	13.4 ± 0.5
3	DL-methionine, with ethionine	0.63	6.6 ± 0.8
3	Glycine, with ethionine	1.23	11.9 ± 0.5
3	DL-homocysteine, with ethionine	1.23	12.8 ± 0.5

* Female rats (170-200 g); fasted 12 hours before treatment, sacrificed 12 hours after start of treatment. 1.23 mMol ethionine given intraperitoneally in four equal doses at 2.5 hour intervals. Test substance given simultaneously with the ethionine.

Male rats were much less susceptible to this effect of ethionine. The ethionine fatty liver could be prevented by methionine, but not by any other amino acid tested. Choline, dimethylthetin, and inositol were also ineffective though homocysteine plus dimethylthetin appeared to have some effect. Large doses of glucose or sucrose given by stomach tube could also prevent the rise in liver lipid.

These observations were extended and confirmed by Jensen *et al.*[26] and by Levine and Fopeano.[27] The latter workers also observed, in addition to the effect on lipid metabolism, that the liver protein content of both male and female rats receiving ethionine and no choline was significantly higher than that of pair-fed controls. Choline administration abolished this difference.

Farber and Segaloff[28] studied the effects of various hormones on the ethionine induced fatty liver in ovariectomized rats. It was found that treatment of the animals with androgens and with growth hormone before giving the ethionine prevented the production of fatty liver to a large degree. Estradiol-17β, 11-deoxycorticosterone acetate, thyroid-stimulating hormone, prolactin, and crystalline zinc insulin were not protective whereas cortisone acetate and corticotropin (ACTH) intensified the fatty liver. Sodium L-thyroxine gave equivocal results.

It is clear that the ethionine-induced fatty liver cannot be due to a deficiency of labile methyl groups, but is the result of a more complex cause. The available evidence indicates that some other aspect of methionine metabolism is importantly involved which has to do with the kind and amounts of protein synthesized. In this regard, it is pertinent to point to the work reported by Singal *et al.*[29] and by the Wisconsin group[30-33] concerning the production of fatty livers in rats by feeding diets in which the amino acids were "out of balance."

However, ethionine is capable of interfering with transmethylation from methionine[34] as is indicated in Table VII. Stekol and Weiss[35] obtained evidence which indicated that ethionine is de-ethylated *in vivo*. Ethionine, labeled with C^{14} in the methylene carbon of the ethyl group, was administered to rats and radioactive creatinine and choline were isolated from the tissues. The radioactivity was confined to the trimethylamine moiety of the molecule. In addition, when ethionine-S^{35} was administered to rats together with bromobenzene, radioactive *p*-bromophenylmercapturic acid was isolated from the urine. Schlenk and Tillotson[36] found that *Torulopsis utilis* and

TABLE VII

Inhibition of Choline Synthesis by DL-Ethionine[34]

Rat No.	Diet	% Creatine methyl derived from dietary methionine	% Choline methyl derived from dietary methionine
1	Ethionine	—	7.20 ± 0.10
2	"	6.85 ± 0.18	8.66 ± 0.10
3	Control	5.50 ± 0.18	10.5 ± 0.08
4	"	5.68 ± 0.18	10.4 ± 0.07

Saccharomyces cerevisiae grown in a medium containing DL-ethionine accumulated a sulfur-containing nucleotide which appeared to be 5′-ethylthioadenosine. This biosynthesis resembles the metabolic relation between methionine and methylthioadenosine and is in accord with the idea that ethionine can be deethylated *in vivo*.

In summary, it is clear that the antimetabolites of choline and methionine discussed do antagonize the metabolic effects of their respective metabolites. However, their fundamental mechanisms of action remain undefined.

REFERENCES

1. WELLS, I. C.: Oxidation of choline-like substances. Inhibitors of choline oxidase. *J. Biol. Chem.* 207: 575, 1954.
2. DUBNOFF, J. W. and BORSOOK, H.: Dimethylthetin and dimethyl-β-propiothetin in methionine synthesis. *J. Biol. Chem.* 176: 789, 1948.
3. DUBNOFF, J. W.: The role of choline oxidase in labelizing choline methyl. *Arch. Biochem.* 24: 251, 1949.
4. MUNTZ, J. A.: The inability of choline to transfer a methyl group directly to homocysteine for methionine formation. *J. Biol. Chem.* 182: 489, 1950.
5. WELLS, I. C.: Antimetabolites of choline. Studies in young rats. *J. Biol. Chem.* 217: 631, 1955.
6. GRIFFITH, W. H.: The relation of choline to the kidneys. *Biol. Symposia* 5: 193, 1941.
7. WELCH, A. D.: The relation of the structure of choline-like compounds to renal antihemorrhagic action. *J. Nutrition* 40: 113, 1950.
8. WELLS, I. C.: Choline antimetabolites. Studies in microorganisms. *J. Biol. Chem.* 222: 923, 1956.
9. WELLS, I. C. and MALLOV, S.: Choline antimetabolites. Studies with intestinal muscle. *Proc. Soc. Exper. Biol. & Med.* 93: 81, 1956.
10. WELLS, I. C.: Inhibition of choline synthesis in young rats by 2-amino-2-methylpropanol-1. *J. Biol. Chem.* 222: 931, 1956.
11. GRIFFITH, W. H. and MULFORD, D. J.: Choline metabolism. Hemorrhagic degeneration and the labile methyl supply. *J. Am. Chem. Soc.* 63: 929, 1941.
12. DYER, H. M.: Evidence of the physiological specificity of methionine in regard to the methylthiol group. The synthesis of S-ethylhomocysteine (ethionine) and a study of its availability for growth. *J. Biol. Chem.* 124: 519, 1938.
13. SHEN, C. W. and LEWIS, H. B.: The metabolism of sulfur. The distribution of urinary sulfur and the excretion of keto acids after the oral administration of some derivatives of cystine and methionine to the rabbit. *J. Biol. Chem.* 165: 115, 1946.
14. HARRIS, J. S. and KOHN, H. I.: On the mode of action of the sulfonamides. The specific antagonism between methionine and the sulfonamides in *escherichia coli*. *J. Pharmacol. and Exper. Therap.* 73: 383, 1941.
15. STEKOL, J. A. and WEISS, K.: Study on growth inhibition by D-, L- and DL-ethionine in the rat and its alleviation by the sulfur-containing amino acids and choline. *J. Biol. Chem.* 179: 1049, 1949.
16. SIMPSON, M. V., FARBER, E., and TARVER, H.: Studies on ethionine. Inhibition of protein synthesis in intact animals. *J. Biol. Chem.* 182: 81, 1950.
17. KIHARA, H. and SNELL, E. E.: Peptides and bacterial growth. Relation to inhibitions by thienylalanine, ethionine and canavanine. *J. Biol. Chem.* 212: 83, 1955.
18. WU, C. and BOLLMAN, J. L.: Effect of ethionine on the free amino acids in the rat. *J. Biol. Chem.* 210: 673, 1954.
19. LEVY, H. M., MONTANEZ, G., and DUNN, M. S.: Effect of ethionine and fasting on the free amino acids of rat liver. *J. Biol. Chem.* 212: 985, 1955.
20. LEVINE, M. and TARVER, H.: Studies on ethionine. Incorporation of ethionine into rat proteins. *J. Biol. Chem.* 192: 835, 1951.
21. RABINOVITZ, M., OLSON, M. E., and GREENBERG, D. M.: Characteristics of the inhibition by ethionine of the incorporation of methionine into proteins of the Ehrlich ascites carcinoma *in vitro*. *J. Biol. Chem.* 227: 217, 1957.
22. GROSS, D. and TARVER, H.: Studies on ethionine. The incorporation of ethionine into the proteins of *Tetrahymena*. *J. Biol. Chem.* 217: 169, 1955.
23. ZAMECNIK, P. C. and KELLER, E. B.: Relation between phosphate energy donors and incorporation of labeled amino acids into proteins. *J. Biol. Chem.* 209: 337, 1954.
24. SIDRANSKY, H. and FARBER, E.: The effects of ethionine upon protein metabolism in the pancreas of the rat. *J. Biol. Chem.* 219: 231, 1956.
25. FARBER, E., SIMPSON, M. V., and TARVER, H.: Studies on ethionine. The interference with lipide metabolism. *J. Biol. Chem.* 182: 91, 1950.
26. JENSEN, D., CHAIKOFF, I. L., and TARVER, H.: The ethionine induced fatty liver: Dosage, prevention and structural specificity. *J. Biol. Chem.* 192: 395, 1951.
27. LEVINE, M. and FOPEANO, J. V., JR.: Effect of ethionine on the protein content of liver in growing rats. *J. Biol. Chem.* 202: 597, 1953.
28. FARBER, E. and SEGALOFF, A.: Effect of androgens and growth and other hormones on ethionine

fatty liver in rats. *J. Biol. Chem.* 216: 471, 1955.

29. SINGAL, S. A., HAZAN, S. J., SYNDENSTRICKER, V. P., and LITTLEJOHN, J. M.: The production of fatty livers in rats on threonine- and lysine-deficient diets. *J. Biol. Chem.* 200: 867, 1953. The effect of threonine deficiency on the synthesis of some phosphorus fractions in the rat. *J. Biol. Chem.* 200: 875, 1953. The lipotropic action of threonine and related substances in the rat. *J. Biol. Chem.* 200: 883, 1953.
30. HARPER, A. E., BENTON, D. A., WINJE, M. E., MONSON, W. J., and ELVEHJEM, C. A.: Effect of threonine on fat deposition in the livers of mature rats. *J. Biol. Chem.* 209: 165, 1954.
31. HARPER, A. E., BENTON, D. A., WINJE, M. E., and ELVEHJEM, C. A.: On the lipotropic action of protein. *J. Biol. Chem.* 209: 171, 1954.
32. BENTON, D. A., HARPER, A. E., WINJE, M. E., and ELVEHJEM, C. A.: Compounds other than threonine that affect fat deposition in the livers of rats fed low protein diets. *J. Biol. Chem.* 214: 677, 1955.
33. ARATA, D., SVENNEBY, G., WILLIAMS, J. N., JR., and ELVEHJEM, C. A.: Metabolic factors and the development of fatty livers in partial threonine deficiency. *J. Biol. Chem.* 219: 327, 1956.
34. SIMMONDS, S., KELLER, E. B., CHANDLER, J. P., and DU VIGNEAUD, V.: The effect of ethionine on transmethylation from methionine to choline and creatine *in vivo*. *J. Biol. Chem.* 183: 191, 1950.
35. STEKOL, J. A. and WEISS, K.: On deethylation of ethionine in the rat. *J. Biol. Chem.* 185: 577, 1950.
36. SCHLENK, F. and TILLOTSON, J. A.: Formation of 5′-ethylthioadenosine from DL-ethionine in yeast. *J. Biol. Chem.* 206: 687, 1954.

DISCUSSION

Dr. R. Bentley (University of Pittsburgh, Pittsburgh, Pa.): I think Dr. Wells would agree that the work he has presented this afternoon indicates once again the very intricate and frequently confusing interrelationships which exist among these various lipotropic factors. In this case we have, on the one hand, two inhibitors which *in vitro* inhibit the choline dehydrogenase system. *In vivo*, on the other hand, these compounds do not inhibit the growth of rats on methionine-deficient diets which are fed with choline and homocystine. Apparently the inhibitory action on the choline dehydrogenase system is no longer manifested *in vivo*. I wonder if Dr. Wells knows at which point the *in vitro* inhibition takes place, since I think it is very important for the present discussion.

I would like to consider possible reasons for this failure of the inhibitors to act *in vivo*. One explanation may be that the inhibitors are transformed to other compounds which no longer are inhibitors of the choline dehydrogenase system but which do seem to have this second antagonistic relationship to choline. Apparently Dr. Wells can rule out methylation as a transforming reaction here, but I wonder perhaps if he has any information about the metabolic fate of, for example, 2-amino-2-methylpropanol. Are there any experiments to work out what happens to this compound? This may be an important point for exploration in order to define precisely the compound which is antagonistic with choline in the second type of reaction that Dr. Wells was discussing.

Another possibility is that it is incorrect to look for antagonisms between these inhibitors or their metabolites, and choline. I was wondering if we might not find another possible mechanism for the action of these inhibitors, particularly the 2-amino-2-methylpropanol. One possible antagonism might be seen with the 1-amino-2-propanol moiety present in vitamin B_{12}. This may sound like an unusual idea, but perhaps it is worth throwing out.

In the amino compounds used as inhibitors and in the aminopropanol present in B_{12}, the amino groups and hydroxyl groups are quite similar. They are both separated by two carbon atoms. I think it is possible that the added 2-amino-2-methylpropanol might undergo phosphorylation, for example, and the analogy would then be even closer with the chemical situation in vitamin B_{12}. If I understand the discussion this morning correctly, I think that Dr. Stekol is not convinced that it is possible to pin down a role for vitamin B_{12} in the *de novo* synthesis of methyl groups. On the other hand, there seems to be sufficient evidence of a connection between vitamin B_{12} and choline synthesis, and I wonder if it might be worth considering that 2-amino-2-methylpropanol is, in effect, antagonistic toward vitamin B_{12}.

Dr. Stekol: I have two comments. The first is that 2-amino-2-methylpropanol is an analogue of dimethylaminoethanol. I think it more profitable to consider the effect of 2-amino-2-methylpropanol as an antagonist to dimethylaminoethanol. Labeled 2-amino-2-methyl propanol is incorporated into tissue phospholipid. It is apparently methylated by methionine. That is my first comment.

The second comment is apropos of ethionine. Ethionine has been accused of being an antagonist of methionine in the protein synthesis by being incorporated into the protein in positions where methionine usually goes. Quantitative considerations, however, rule that theory out. The quantities of ethionine incorporated are of the order of 10^{-3} of the extent of incorporation of methionine. However, the idea that ethionine antagonizes methionine in protein synthesis could be looked upon from the standpoint of metabolism of methionine and ethionine, particularly with respect to the involvement of ATP in the metabolism.

Recently, we have reported the isolation of S-adenosyl-ethionine after incubation of radioethionine

with ATP in liver preparations. The amounts of S-adenosyl-ethionine and S-adenosyl-methionine formed by the liver are about equal. That means that ethionine and methionine would compete for ATP.

The competition for ATP between the two amino acids appears to explain practically every physiological effect of ethionine that one observes in the intact animal, first, the fatty infiltration; second, the alleviation of the so-called fatty infiltration by glucose; third, the inhibition of glucose oxidation upon administration of ethionine; fourth, the lowering of the capacity of the animal to synthesize choline from methionine after the injection of ethionine; fifth, complete destruction, within six to twelve hours, of acinar cells of the pancreas after ethionine administration; sixth, inhibition of "protein synthesis," as studied by the uptake of radioactive amino acids, and so on. We believe, therefore, that the antimetabolic effect of ethionine is due simply to stoichiometric competition of ATP between methionine and ethionine.

The compound that Dr. Lewis of the University of Michigan, reported some years ago as being excreted in the urine of the rat in apparently unchanged form after feeding ethionine is S-adenosyl-ethionine which can be readily isolated from the urine. S-adenosyl-ethionine does not act as a donor of ethyl group as actively as S-adenosyl-methionine acts as the donor of its methyl. It looks, then, that "binding" of ATP by ethionine is irreversible in a physiologic sense, whereas this "binding" by methionine is not. For that reason ethionine would constitute a drain on the ATP of the animal organism.

Dr. Wells failed to mention, and I think it is a very important question, that the continuous administration of ethionine to rats orally or intraperitoneally, produces after 11 months, carcinoma of the liver. As we reported, I think, at the last meeting, we have been following the changes in the lactic acid production in rats with time after a single administration of ethionine. There is an increase in lactic acid synthesis by the liver; an inhibition of glucose oxidation to CO_2; there is an inhibition of pyruvate oxidation which is not due to inhibition of thioctic acid synthesis or to any other alteration except in the extent of synthesis of coenzyme A. This again could be correlated with the inactivation of ATP by ethionine. From that standpoint, if you look at the metabolic picture of ethionine, it will be a little easier, perhaps, to understand the various physiologic effects induced by ethionine which have been discussed here.

Dr. Artom: I would like to ask a question of Dr. Stekol. Does he know whether 2-methyl-2-amino-propanol is methylated before or after incorporation into a phospholipid? I am asking this question, because we have shown that *in vivo* as well as *in vitro* dimethylethanolamine can be incorporated into a phospholipid even before being further methylated to choline.

Dr. Stekol: I cannot say what happens first, whether it was incorporated first and then methylated or whether it was methylated and then incorporated. All I can say is that 2-amino-2-methyl moieties of 2-amino-2-methylpropanol are in phospholipid which could be detected chromatographically. Apparently the compound is in the methylated form. Proof of the pudding will be the isolation of the methylated 2-methyl-2-aminopropanol. This we have not done yet. That is the reason why I feel that this discussion be sort of "off the record."

Dr. A. L. Sheffner (Mead Johnson & Company, Evansville, Ind.): Does the injection of ethionine affect other enzymatic reactions like transamination or production of urea?

Dr. Stekol: We have studied four or five. There was a report, I forget by whom, on the inhibition of amylase synthesis by ethionine, but that has not been confirmed, and on the inhibition of choline oxidase. There is apparently an inhibition of many enzymatic reactions *in vivo*, by ethionine administration but any reaction that involves coenzyme A or ATP will be in trouble with ethionine. For that reason a very confusing and widespread effect of ethionine in the whole animal I think could be explained, perhaps, on the basis of the inhibition of coenzyme A formation and involvement of ATP in ethionine metabolism would account for at least some of the inhibitions of some of the enzymatic reactions by ethionine administration to animals.

Dr. I. Wells (closing remarks): The first question concerned the metabolic fate of 2-amino-2-methylpropanol. I have nothing definite to say on this. Dr. Stekol's comment that this material is incorporated into the phospholipid is very interesting indeed. I do know that apparently this inhibitor as administered disappears fairly rapidly and its administration must be continued in order to get an effect.

As far as the compound being an antagonist of the aminopropanol which has been isolated from vitamin B_{12}, we also at one time thought of this; however, on second thought, we did not know of a definitive role which had been demonstrated for B_{12} to play in methylation reactions and hence we have not investigated it further.

I must apologize to Dr. Stekol. I was not aware of a report that ethionine causes carcinoma in rats.

Dr. Stekol: That was reported by Farber about five years ago.

Dr. Wells: I would like to ask Dr. Stekol one question about his explanation for ethionine action. I still am not clear how this would explain the sex difference which has been observed.

Dr. Stekol: I do not believe the sex difference has been confirmed by the same workers who originally claimed it.

Dr. K. Hofmann (University of Pittsburgh, Pittsburgh,

Pa.): We believe that we understand the structure of lipids as they are nicely categorized in all the textbooks, and we think we know what lipids really are. I would like to support the point of view that we really have just begun to scratch the surface regarding lipid structure. I don't think that we have any information on the state of the lipids in lipoproteins and how they are linked to the protein. Also, little is known about the complex lipid structures which constitute the cell walls.

I think that questions regarding lipid metabolism which we are unable to answer based on present-day knowledge, might be answered if we do not neglect further study on the chemical structure of the more complex substances which constitute combinations of carbohydrates and lipids, and combinations of proteins and lipids. This is an area in which there is complete ignorance at the present time, and I think we should explore these aspects of the problem in conjunction with inquiries into the metabolic or nutritional aspects of lipids.

The Renal Lesions in Choline Deficiency

WENDELL H. GRIFFITH, PH.D.*

THE RECOGNITION that a renal lesion is one of the direct effects of a deficiency of choline in the diet of weanling rats was the result of a study of the role of amino acids as lipotropic compounds.[1] It had been my good fortune to be a student of the late Howard B. Lewis and, as was true of so many of those he trained, a very real interest in the metabolic relationships of the structural components of the proteins had been kindled by his zeal and enthusiasm for research in this field.[2] The stimulation to investigate the specific effects of amino acids on the development of the "fatty liver" resulted from hearing H. J. Channon discuss some of his experimental findings on this subject at a meeting of the British Biochemical Society at University College, London, early in 1937.[3] His paper was an extension of the earlier report[4] on the surprising effect of cystine in increasing the deposition of liver fat in adult rats on a low-choline diet. This report was followed within the year by the beautiful demonstration by Tucker and Eckstein that methionine, the other principal sulfur-containing amino acid of proteins, exerted an opposite effect.[5] At this time no information was available on the phenomenon of transmethylation, nor was there any real hint regarding the *de novo* synthesis of methyl groups.

The experimental procedures of Beeston and Channon and of Tucker and Eckstein followed the pattern set by Best and Huntsman[6] who in 1932 first demonstrated the lipotropic action of choline in rats and who in 1935 showed that casein also has lipotropic properties. Channon and Wilkinson confirmed this finding and introduced the low-casein (5 per cent), high-fat (40 per cent) diet which was widely adopted in the study of fatty liver production in older rats.[7]

The decision to study this problem by using young rather than adult rats was, of course, based on the anticipation that the additional requirements of both choline and amino acids for growth might permit a clearer definition of their interrelations. A deliberate effort was made, therefore, to select a combination of proteins of the highest biologic value in order to provide the maximum opportunity for growth and, at the same time, the maximum fatty liver effect, it being assumed that low total protein favored the accumulation of lipids in the liver. Accordingly, fibrin, casein, and ovalbumin were selected as proteins that supplied a superior mixture of amino acids, at least for growth, and the effectiveness of the diet was tested first with growth and not choline deficiency in mind. It is pertinent to note that at this date it was not feasible to use mixtures of pure amino acids in place of protein and that the adequacy of the supply of water-soluble vitamins could not be guaranteed without the use of extracts of liver, of rice "polish," or of yeast. Dried hog liver (2 per cent) and powdered brewer's yeast (5 per cent) were added to these original diets to ensure, if possible, the provision of trace nutrients. As in the studies of Best and others on older rats the diet was high in fat (40 per cent). Diets containing the three proteins, singly and in various combinations, were fed weanling rats for a 30-day period.[8]

Two unexpected findings resulted from this first experiment (Table I). Extra liver fat was found in every rat despite the significant amounts of choline provided by the liver and yeast supplements. Furthermore, the extent of the fatty liver was proportional to the level of dietary protein and to the rate of growth. The

From the Department of Physiological Chemistry, University of California Medical Center, Los Angeles.
* Professor and Chairman.

Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc., New York, New York.

TABLE I

Production of Fatty Livers in Weanling Male Rats

Total dietary protein* g/100 g	Added choline chloride g/100 g	Gain in weight g	Total liver weight g	Total liver lipids g	Lipids in liver %
5	0	21	3.70	1.02	27.5
10	0	59	5.50	1.50	27.3
15	0	67	7.58	2.32	30.6
5	0.5	38	3.35	0.18	5.4
10	0.5	65	4.50	0.21	4.7
15	0.5	71	4.75	0.28	6.0

* Fibrin:casein:egg albumin, 2:2:1 (modified from Griffith and Wade[8]).

livers of the rats fed the 15 per cent protein mixture were especially enlarged and grossly fatty in appearance. The heavy deposition of hepatic fat (30.6 per cent), which was largely prevented by a dietary supplement of choline, demonstrated clearly that weanling rats were indeed likely experimental animals for these studies because changes in liver lipids could be produced regardless of the adequacy of the dietary protein for growth.

Following this demonstration, a similar diet was prepared in which the choline-containing liver and yeast supplements were replaced by a mixture of pure vitamins and of aqueous concentrates of rice "polish" and hog liver. Autopsy of a group of weanling male rats after a 10-day period on the modified diet showed greatly enlarged, purplish-red kidneys with hemorrhagic capsules in 39 of 40 rats as shown in Figure 1. No such lesion was observed in a group fed the same diet supplemented with 0.4 mg of choline chloride per gram of food, but the livers were extremely fatty. The addition of 2.0 mg of choline chloride per g of food was required to prevent the deposition of extra hepatic lipids as well as the renal pathology. Needless to say, the acceptance of the renal lesion as an effect of choline deficiency was not immediate. After the first observations all dietary components were discarded, including the salt mixture, and new diets were prepared with care to avoid accidental contamination with toxic substances. However, the lesions were produced consistently and were just as consistently prevented by small supplements of choline.

The renal lesion was called "hemorrhagic degeneration" because of the unmistakable evidence of subcapsular bleeding and because of the hemorrhagic appearance of the cortex of the kidney. In a typical series the characteristic sequence included the maximal deposition of liver lipids during the first five days, elevation of the blood nonprotein nitrogen on the fifth day and uremic levels of approximately 250 mg per 100 ml on the seventh day. Renal function at this time was markedly impaired.[9] More severely affected animals showed ocular hemorrhage also. Mortality varied from 0 to 100 per cent depending on such factors as the completeness of the removal of choline from the diet, the age and sex of the rats and, as was shown later,[10,11] the amount of dietary sulfur and its distribution between cystine and methionine. Hemorrhagic degeneration was wholly pre-

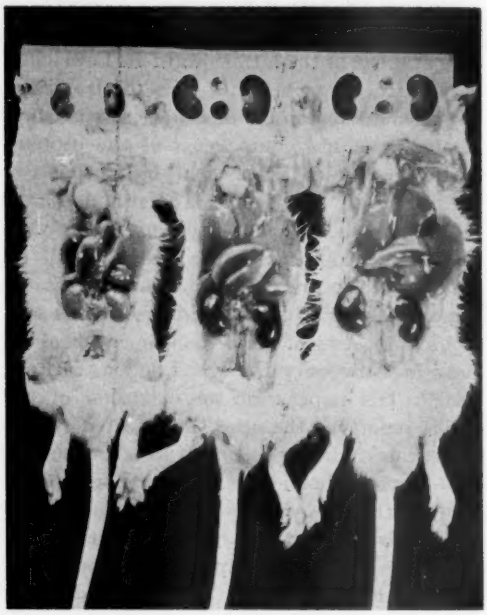


Fig. 1. Effects of choline deficiency in weanling male rats after eight days on low-choline diet. The control rat is on the left. The center rat is in the acute phase of hemorrhagic degeneration. The rat on the right is in the first stage of the recovery phase. The kidneys at the top of the plate illustrate the characteristic cortical congestion of the acute lesion. The marked fatty enlargement of the liver and hemorrhage into the eyeballs (placed between the kidney sections) are evident.

vented by supplements of choline too small to affect the deposition of liver lipids.

The increase in weight and the gross appearance of the affected kidneys afforded criteria of the severity of the choline-deficient diet. These changes, which have been described by Christensen,[12] were due primarily to the congestion of the cortical capillaries and capsular vessels. Peripheral glomeruli frequently appeared distended with blood but red cells were not observed in the tubules. Hemorrhage was slight except in the capsule and at the periphery of the cortex. More severely affected kidneys exhibited massive destruction of the vascular system in this region (Fig. 2).

The most significant degenerative change, however, was in the renal tubules. Affected parts of convoluted tubules in the cortex showed necrosis, albuminous degeneration, or hyaline droplet degeneration. Edema and desquamation were observed in some. Tubules in the deep part of the cortex and in the outer portion of the medulla were always filled with casts. Numerous fat droplets were demonstrable histologically (Scharlach R) in the necrotic tubular cells as well as in the blood of the congested cortical vessels.

Almost as spectacular as the onset of hemorrhagic degeneration was the spontaneous recovery observed in many animals surviving the acute phase. As the cortical congestion disappeared, the kidneys remained enlarged and

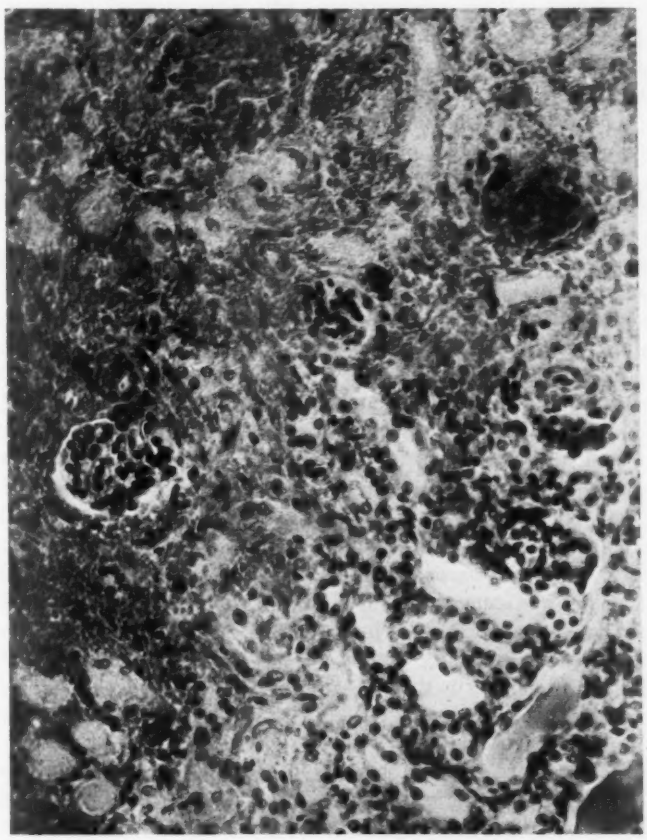


Fig. 2. Peripheral zone of the renal cortex during the acute stage of choline deficiency in weanling rats, showing cortical congestion and tubular necrosis (hematoxylin and eosin × 165).

light yellow in color, if the previous acute stage was moderate, but chalky, yellowish-white if the acute stage was severe. Kidneys of the latter type were frequently covered with irregular white deposits and were described as "frosted" as noted in one animal shown in Figure 1. Kidneys in recovered animals examined months after the acute phase were usually of normal weight, sallow brown in color, irregular in shape and roughened by grooves or depressions which marked the location of scar tissue.

Why does hemorrhagic degeneration occur? Hartroft and Best have suggested the following sequence of events which may account for the histologic alterations in the renal structure.[13,14] Small droplets of stainable fat appear in the epithelial cells of the proximal convoluted tubules of the cortex; concomitantly, generalized swelling of the affected nephrons occurs, with obstruction of the intervening plexus in the cortex; tubular ischemia and necrosis result and there is engorgement of the proximal cortical vessels which rupture into and beneath the capsule. Whether or not fat droplets are causal agents for subsequent events is, of course, uncertain. Baxter and Goodman were unable to demonstrate an increase in renal lipids prior to the onset of the degeneration and have expressed caution in concluding that the appearance of the fat is other than a manifestation of the pathologic process.[15] In any event, the question of the origin of the biochemical alterations remains unanswered.

An unproved but reasonable working hypothesis involves the concept that the kidney requires a continuing source of choline for the synthesis of lecithin. The biogenesis of lecithin has been clarified by Kennedy who has demonstrated that choline is incorporated into the phospholipid by way of phosphorylcholine and cytidine diphosphate choline in the presence of the enzymes, phosphorylcholine-cytidyl transferase and phosphorylcholine glyceride transferase.[16,17] Cofactors of dietary origin may be required for these enzymatic reactions. However, if it is assumed that the failure in hemorrhagic degeneration is in the availability of choline rather than in its conversion into lecithin, four sources of choline, at least, must be taken into account, viz., dietary choline, choline cleaved from choline-containing tissue constituents, choline formed by transfer of methyl from a methyl donor, such as methionine, to dimethylethanolamine, and choline formed from ethanolamine by the *de novo* synthesis of methyl from one-carbon fragments. Most of the investigations of choline deficiency have been concerned with the dietary supply of choline itself, of the methyl donors, methionine and betaine, of the methyl acceptors, ethanolamine and its mono- and dimethyl derivatives, and of the trace nutrients required in transmethylation and in formate-to-methyl synthesis, notably folic acid and vitamin B_{12}.

Consideration of some of these relationships is illustrated in a recent paper by Young *et al.*[18] who have studied the relative extent of biogenesis of choline by *de novo* synthesis of methyl and by transmethylation in young rats. Striking, in this connection, were the findings that monomethylethanolamine prevented renal lesions in the absence of vitamin B_{12}, that the vitamin was ineffective in this regard in the absence of the ethanolamine derivative, that the vitamin was not required for the transfer of methyl from betaine, and that it did favor *de novo* synthesis of methyl to compensate for a partial deficiency of preformed dietary methyl.

Further evidence that the nonavailability of choline is the primary cause of hemorrhagic degeneration has been provided by Wells who has demonstrated that 2-amino-2-methylpropanol-1 and α,α-dimethyltriethylcholine act as competitive inhibitors of the utilization of choline in the kidney and that the renal lesion is increased by their administration.[19] Conceivably, such an inhibition might be effective by interference with ethanolamine in the *de novo* synthesis of methyl, by interference with dimethylethanolamine in its role as an acceptor of methyl by transmethylation, or by interference with choline in the reaction in which lecithin is formed.

How do these possibilities fit the observations that have been made with regard to the experimental factors affecting the incidence and severity of the renal lesion? Obviously, the ease of production of the lesion in weanling male rats permits the conclusion that the re-

quirement of choline is relatively large at this time, larger than can be compensated by the usual reactions of biogenesis. Furthermore, the rapid recovery in surviving animals, without dietary change, suggests either that the need of choline is temporary only, which is improbable, or that adaptive synthetic mechanisms provide an additional supply. If such is the case, it is noteworthy that the additional supply is insufficient to reduce appreciably the level of the fatty liver.

It was apparent early in the investigation of hemorrhagic degeneration that its severity was related to the age of the rats.[20,21] Using a constant starting weight of 40 g, 20-day-old males showed a maximum incidence of lesions but very low mortality. Furthermore, most of these rats were in the recovery phase at the end of the 10-day period. Rats placed on the diet from ages 21 to 27 days showed maximum lesions and high mortality, with the most severe lesions in rats 23 days old at the start. On the other hand, if the rats remained on the stock diet until they were past 30 days of age, much more drastic procedures were required to produce severe renal lesions. Because of the astonishing difference in the severity of the choline deficiency in 20 and 23 day-old rats, it was suggested that a protective substance was present in milk, inasmuch as the 20-day old rats were suckling up to the time the experimental feeding began. Interestingly, Mulford has reported recently that weanling rats are less susceptible to choline deficiency if vitamin B_{12} is administered to the mother during the lactation period.[22]

In general, the severity of the deficiency of choline in young rats has been found to be consistent with the data in Table I, i.e., the more adequate the diet for growth, except for its provision of choline or of the dietary essentials for the formation of choline, the more severe is the result of the deficiency. For example, the effect of cystine in aggravating the severity of the renal lesion has been explained as due to its effect in removing a dietary lack of sulfur.[11] Of especial significance was the demonstration that restriction of caloric intake also decreased the need of choline, presumably by impairment of growth.[11] Thus, care is needed to distinguish between protection against renal lesions due to a choline-like effect of a test substance and that due to a depressing effect of the test substance on the food intake of experimental rats on a low-choline diet.

Pertinent to the intriguing susceptibility of the young male rat to a deficiency of choline when approximately 30 days of age is Chanutin's concept of chemical maturity in this species.[23] Whereas the dried, fat- and ash-free tissue of young rats increases progressively from birth, Chanutin found that the percentage of creatine in this body component increased sharply from about 0.85 per cent on the 20th day of age to 1.10 per cent on the 30th. Thus, the concentration of creatine in the tissues of the rat reaches a maximum at this time and a deficiency of methyl may be particularly critical. Furthermore, Kensler *et al.*[24] have shown that choline oxidase of the liver and kidneys increases sharply during this same period. Regardless of these factors, occurrence of the lesion has been noted in older rats. Handler[25] showed that the remaining kidney in adult rats became hemorrhagic if the animals were placed on a methyl-deficient diet immediately after unilateral nephrectomy. Mulford[26] has produced the lesion within 9 to 13 days in rats 9 to 20 weeks of age by the addition of 20 mg of 2-amino-2 methyl-propanol-1 per gram of food. Renal damage was observed in older rats, including female rats, on low-choline diets containing added cholesterol.[21,27] Growth hormone likewise aggravated the choline deficiency.[28,29]

It is apparent that among these reports no specific unequivocal explanation has been formulated to account for the renal lesion, although a great deal of information has been obtained on the facts associated with its occurrence. What of the studies in which measurements have been made of relevant constituents of kidney tissue? Jacobi and Baumann determined the choline content of rat kidneys and of other tissues during the development of the renal lesion and found no evidence that choline synthesis did not proceed normally during the critical period.[30] They suggested that the symptoms of choline deficiency are due to a lack of an as yet unidentified methyl-contain-

ing essential other than choline itself. On the other hand, Patterson and McHenry[31] have provided evidence of a direct link between a decrease in phospholipid in the kidneys and the onset of renal disease. The percentage concentration of phospholipid in the kidneys and livers of choline-deficient rats was markedly reduced below the concentration of phospholipid in the kidneys and livers of choline-fed animals. The reduction in the kidney during the first seven days of the experimental period was an absolute decrease, i.e., phospholipid was lost from the kidney. During the 10 days in which rats were maintained on the choline-free diet, the kidney weight doubled due to hemorrhagic degeneration, the total lipid increased by 28 per cent, the total nitrogen by 51 per cent but the total phospholipid decreased slightly. After the first seven days, prior to the appearance of the lesion and when the kidney weight, total fat, and total nitrogen were approximately the same in the deficient and control animals, the total phospholipid was reduced to 75 per cent of the level at the start of the experiment.

Reference has been made earlier to the possibility that the appearance of stainable fat in renal cells is related to the onset of hemorrhagic degeneration. Best and co-workers are of the opinion that many tissues other than the liver become fatty, to a lesser extent to be sure, during choline deficiency and that choline is a lipotropic substance for these tissues as well as the liver. Moore[32] has noted fatty changes in the kidneys but was unable to relate these to the necrotic lesions. It is significant that the nature of the fatty acids of the diet has been demonstrated to influence the development of renal lesions. Engel[33] believes that both pyridoxine and essential fatty acids are required for the lipotropic action of choline. Both Stetten and Salcedo[34] and Daft[35] observed increased severity of choline deficiency if the diet contained saturated fatty acids.

Of interest is the observation of Ryan and Wade[36] that the predominately filamentous mitochondria of cells in the renal cortex were replaced by granular types during choline deficiency. The filamentous form was restored after the return of the animals to the control diet. Olson and Deane[37] had previously noted the fragmentation of mitochondria in the tubular epithelium and the decreased respiration of slices of cortex of degenerated kidneys. Fischer and Garrity[38] have reported a correlation between the appearance of renal lesions, a decrease in serum albumin, and an increase in α-globulin in weanling rats. They interpreted their findings as indicating that priority of use of methionine for the synthesis of serum albumin exaggerated the lack of methyl and of choline and precipitated the renal lesion.

This presentation has been limited to the more prominent observations regarding renal lesions in rats during a dietary deficiency of choline. No attempt has been made to include references to the many important papers concerned with additional nutritional aspects of the syndrome, with choline deficiency in other species, or with effects in rats which appear subsequent to a pre-existing lesion, such as the studies by Best, *et al.* on experimental hypertension,[8] on serum lipoproteins,[39] and on cardiovascular abnormalities.[40] In none of these investigations are analytical data presented on variations in levels of enzymes and of substrates in renal tissue that permit the portrayal of the series of chemical events leading over a period of only a few days to remarkable changes in the mass and the character of the kidney, changes that are all the more striking because they may include both degeneration and recovery.

It may be said that for approximately two decades hemorrhagic degeneration has been serving a useful purpose as an easily recognized indicator of the severity of a deficiency of choline and of nutrients concerned with the biogenesis of choline in weanling male rats. For nearly one decade the lesion has been used in the study of experimental hypertension in older rats. Its usefulness in these respects should not mask the incompleteness of our knowledge of the mechanisms that are involved, and of the possible applications in medical science this knowledge might yield. One would be remiss, indeed, not to emphasize the unique opportunity afforded by the renal lesion of choline deficiency for fruitful research.

REFERENCES

1. GRIFFITH, W. H. and WADE, N. J.: Some effects of low choline diets. *Proc. Soc. Exper. Biol. & Med.* 41: 188, 1939.
2. GRIFFITH, W. H. and LEWIS, H. B.: Studies in the synthesis of hippuric acid in the animal organism. V. The influence of amino-acids and related substances on the synthesis and rate of elimination of hippuric acid after the administration of benzoate. *J. Biol. Chem.* 57: 1, 1923.
3. BEESTON, A. W., CHANNON, H. J., and PLATT, A. P.: Amino acids and production of fatty livers. *J. Soc. Chem. Ind.* 56: 292, 1937.
4. BEESTON, A. W. and CHANNON, H. J.: XLIV. Cystine and the dietary production of fatty livers. *Biochem. J.* 30: 280, 1936.
5. TUCKER, H. F. and ECKSTEIN, H. C.: The effect of supplementary methionine and cystine on the production of fatty livers by diet. *J. Biol. Chem.* 121: 479, 1937.
6. BEST, C. H. and HUNTSMAN, M. E.: The effects of the components of lecithine upon deposition of fat in the liver. *J. Physiol.* 75: 405, 1932; The effect of choline on the liver fat of rats in various states of nutrition. *J. Physiol.* 83: 255, 1935.
7. CHANNON, H. J. and WILKINSON, H.: XLII. Protein and the dietary production of fatty livers. *Biochem. J.* 29: 350, 1935.
8. GRIFFITH, W. H. and WADE, N. J.: Choline metabolism. I. The occurrence and prevention of hemorrhagic degeneration in young rats on a low choline diet. *J. Biol. Chem.* 131: 567, 1939.
9. GRIFFITH, W. H.: The relation of choline to the kidneys. *Biol. Symp.* 5: 193, 1941.
10. GRIFFITH, W. H. and WADE, N. J.: Choline metabolism. II. The interrelationship of choline, cystine, and methionine in the occurrence and presentation of hemorrhagic degeneration in young rats. *J. Biol. Chem.* 132: 627, 1940.
11. MULFORD, D. J. and GRIFFITH, W. H.: Choline metabolism. VII. The relation of cystine and of methionine to the requirement of choline in young rats. *J. Nutrition* 23: 91, 1942.
12. CHRISTENSEN, K.: Renal changes in the albino rat on low choline and choline-deficient diets. *Arch. Path.* 34: 633, 1942.
13. HARTROFT, W. S.: Pathogenesis of renal lesions in weanling and young adult rats fed choline-deficient diets. *J. Exper. Path.* 29: 483, 1948.
14. BEST, C. H. and HARTROFT, W. S.: Nutrition, renal lesions and hypertension. *Fed. Proc.* 8: 610, 1949.
15. BAXTER, J. H. and GOODMAN, H.: Renal and hepatic lipid alterations in choline deficiency: Relationship to renal necrosis. *Proc. Soc. Exper. Biol. & Med.* 89: 682, 1955.
16. KENNEDY, E. P. and WEISS, S. B.: The function of cytidine coenzymes in the biosynthesis of phospholipides. *J. Biol. Chem.* 222: 193, 1956.
17. BORENHAGEN, L. F. and KENNEDY, E. P.: The enzymatic synthesis of cytidine diphosphate choline. *J. Biol. Chem.* 227: 951, 1957.
18. YOUNG, R. J., LUCAS, C. C., PATTERSON, J. M., and BEST, C. H.: The role of dietary betaine and vitamin B_{12} in choline formation by the rat. *J. Biol. Chem.* 224: 341, 1957.
19. WELLS, I. C.: Antimetabolites of choline. Studies in young rats. *J. Biol. Chem.* 217: 631, 1955; Inhibition of choline synthesis in young rats by 2-amino-2-methylpropanol-1. *J. Biol. Chem.* 222: 931, 1956.
20. GRIFFITH, W. H.: Choline metabolism. IV. The relation of the age, weight and sex of young rats to the occurrence of hemorrhagic degeneration on a low choline diet. *J. Nutrition* 19: 437, 1940.
21. GRIFFITH, W. H. and MULFORD, D. J.: Choline metabolism. VII. Some dietary factors affecting the incidence and severity of hemorrhagic degeneration in young rats. *J. Nutrition* 21: 633, 1941.
22. MULFORD, D. J.: Diet of adult female rats and relation to kidney lesions in choline deficient offspring. *Proc. Soc. Exper. Biol. & Med.* 88: 177, 1955.
23. CHANUTIN, A.: The influence of growth on a number of constituents of the white rat. *J. Biol. Chem.* 93: 31, 1931.
24. KENSLER, C. J., RUDDEN, M., SHAPIRO, E., and LANGEMANN, H.: Choline oxidase activity in young rats. *Proc. Exper. Biol. & Med.* 79: 39, 1952.
25. HANDLER, P.: Factors affecting the occurrence of hemorrhagic kidneys due to choline deficiency. *J. Nutrition* 31: 621, 1946.
26. MULFORD, D. J. and OUTLAND, C. E.: The effect of 2-amino-2-methyl-1-propanol on the incidence of kidney lesions in male rats of different ages fed diets low in choline. *J. Nutrition* 61: 381, 1957.
27. WILGRAM, G. F. and HARTROFT, W. S.: Pathogenesis of fatty and sclerotic lesions in the cardiovascular system of choline-deficient rats. *Brit. J. Exper. Path.* 36: 298, 1955.
28. HALL, C. E. and BIERI, J. G.: Modification of the choline-deficiency syndrome in the rat by somatotrophin and hydrocortisone. *Endocrinology* 53: 661, 1953.
29. WILGRAM, G. F., BEST, C. H., and BLUMENSTEIN, J.: Effect of growth hormone and testosterone on induction of cardiovascular changes in choline-deficient rats. *Proc. Soc. Exper. Biol. & Med.* 91: 620, 1956.
30. JACOBI, H. P. and BAUMANN, C. A.: The biochemical defect in choline-deficient rats. *J. Biol. Chem.* 142: 65, 1942.
31. PATTERSON, J. M. and MCHENRY, E. W.: Choline and the prevention of hemorrhagic kidneys in the rat. III. Amounts of water, nitrogen, total lipid, and choline in livers and kidneys. *J. Biol. Chem.* 156: 265, 1944.

32. MOORE, H. C.: The acute renal lesions produced by choline deficiency in the male weanling rat. *J. Path. & Bact.* 74: 171, 1957.
33. ENGEL, R. W.: The relation of B-vitamins and dietary fat to the lipotropic action of choline. *J. Nutrition* 24: 175, 1942.
34. STETTEN, D. and SALCEDO, J.: The effect of chain length of the dietary fatty acid upon the fatty liver of choline deficiency. *J. Nutrition* 29: 167, 1945.
35. DAFT, F. S.: Part I. Fatty liver and cirrhosis. Experimental differentiation between liver necrosis and liver cirrhosis and some dietary factors affecting their development. *Ann. New York Acad. Sc.* 57: 623, 1954.
36. RYAN, S. and WADE, N. J.: Choline deficiency and mitochondrial morphology in the rat. *Fed. Proc.* 16: 111, 1957.
37. OLSON, R. E. and DEANE, H. W.: A physiological and cytochemical study of the kidney and the adrenal cortex during acute choline deficiency in weanling rats. *J. Nutrition* 39: 31, 1949.
38. FISCHER, M. A. and GARRITY, G. C.: Protein metabolism in the choline-deficient rat. I. Effect of choline on serum proteins. *J. Biol. Chem.* 204: 759, 1953; Protein metabolism in the choline-deficient rat. II. Effects of age and sex on serum proteins. *J. Biol. Chem.* 206: 345, 1954.
39. WILGRAM, G. F., LEWIS, L. A., and BEST, C. H.: Effect of choline and cholesterol on lipoprotein patterns of rats. *Circulation Res.* 5: 111, 1957.
40. WILGRAM, G. F., HARTROFT, W. S., and BEST, C. H.: Dietary choline and the maintenance of the cardiovascular system in rats. *Brit. M. J.* 2: 1, 1954.

DISCUSSION

Dr. Willoughby Lathem (University of Pittsburgh, Pittsburgh, Pa.): Because of the nature and distribution of the lesions that Dr. Griffith has just shown us, attention has been called to the similarities and differences between these lesions in choline deficiency in rats and a clinical syndrome that we see in patients, namely, bilateral cortical necrosis which occurs in a variety of clinical circumstances. Cortical necrosis arises probably as a result of changes in the vasculature of the kidney, and in most instances changes in small and large vessels can be demonstrated in patients dying with this disorder which are primarily of a thrombotic nature. These of course are absent in the kidneys of choline-deficient rats.

Do these lesions arise as a result of changes in the renal vasculature or do they occur secondarily as the result of some disturbance, metabolic or otherwise, in the renal tubules? Although Dr. Griffith did not comment on this point, it is my understanding that in some animals which have less severe degrees of change than those that were demonstrated just now, changes in the tubules can be seen at an earlier time when there is actually no marked change in the vasculature and little hemorrhage demonstrable under the capsule of the kidney. If this is true, this might suggest that the disease initially, at least, is not primarily one of a change in the renal blood supply but occurs secondary to some change in the tubules. If so, why does it affect principally the cortex of the kidney and not other areas?

Dr. Griffith or Dr. Hartroft, do you have any information concerning the metabolic activity of renal tubules when exposed to various amino acid and choline concentrations? What, for example, is the oxygen consumption and what other biochemical changes occur in kidney cortex slices under various conditions *in vitro*?

It is my understanding that one is unable to produce this in other types of experimental animals, only the rat is susceptible. Why?

We do know that the rat seems to differ from other animals in regard to certain aspects of protein metabolism by the kidney. In some animals we can demonstrate that the renal tubules handle protein that is filtered by the glomeruli in such a manner that it appears as if the tubules are actively metabolizing the protein which appears in the ultrafiltrate. The process appears to involve tubular reabsorption and breakdown of the protein by the tubules into amino acids and polypeptides.

In the rat the amount of protein which appears in the urine appears to differ in the female and in the male, and we have just heard that the lesion of choline deficiency is much more easily produced in the male than in the female. Is it possible that these changes are in some way related to species differences between the rat and other animals and to sex differences, male and female, with regard to the amino acid and protein metabolism of the renal tubules of these animals?

Dr. Griffith: The question of the histopathologic involvement of the renal cortex I am happy to refer to Dr. Hartroft. With regard to the *in vitro* study of metabolic processes in kidney tissue, investigations have been rather meager. Dr. Olson is one of the few who has measured *in vitro* respiration of kidney slices in choline deficiency although there is a fair amount of published work on the turnover of phospholipids in kidney tissue.

Dr. Hartroft: The purpose of this symposium is to attempt to explain modes of action of lipotropic factors in nutrition, rather than to study the end results of the deficiency. But a clear knowledge of the effects of the deficiency on organs is essential before attempting to discover *how* they were produced. In discussing the renal lesion in choline-deficiency, I will therefore emphasize its pathology and pathogenesis leaving other speculations aside.

As Dr. Griffith mentioned, nearly 20 years ago he and Wade (*Proc. Soc. Exper. Biol. & Med.* 41: 188, 1939) discovered that weanling rats fed a low-choline diet frequently died within six to twelve days from a hemorrhagic syndrome involving chiefly the kidneys,

but also less often, thymus, lungs, heart, adrenals and eyes. Lesions in the last named organs are considered by most investigators to be secondary to those in the kidney and will not be described here.

Pathology of the Fully Developed Acute Renal Lesion: Grossly, the affected kidneys are large and red. Their weights are twice normal, their capsules tense, smooth, and shiny and the underlying parenchyma swollen, engorged, and hemorrhagic. I emphasize their large size and swollen tense capsules, as these features are pathogenetically significant. Microscopically, bilateral necrosis selectively involves proximal convoluted tubules in the cortex. Certainly the consensus of those who have studied this lesion (György and Goldblatt, *J. Exper. Med.* 72: 1, 1940; Christensen, *Arch. Path.* 34: 633, 1942; Wachstein, *Arch. Path.* 38: 297, 1944; and Hartroft, *Brit. J. Exper. Path.* 29: 483, 1948) is that glomeruli are but secondarily involved. But Moore (*J. Path. and Bact.* 74: 171, 1957) was impressed by the prominence of glomerular lesions and would like to attach more importance to them than had previous investigators; he is the only one to have attached significance to these glomerular lesions—an important point in considering pathogenesis. But Moore's own data indicate that necrosis of proximal tubules always occurs, whereas glomerular lesions are more inconstant and late.

Hemorrhage gives the kidney its typical appearance and led Dr. Griffith to name the condition "hemorrhagic kidney." Microscopically, hemorrhage is largely confined to subcapsular zones. Red blood cells elsewhere are usually within distended capillaries, thereby representing extreme degrees of stasis rather than hemorrhage. Only after tubular necrosis is full-blown and intervening capillaries have thereby lost much of their support does hemorrhage deeper within the cortex develop. The location of tubular necrosis has been defined clearly and is important in considering pathogenesis. It develops initially at peripheries of renal lobules, involving only proximal convoluted segments. Later, necrosis in other portions of the renal lobule and of other structures (glomeruli and distal convoluted segments of tubules) may be observed, but is clearly secondary to the initial, precise and selective localization of lesions. Obviously, primary and secondary events must be differentiated in these studies.

In fully developed lesions, stainable fat is not a prominent feature in suitably prepared sections. At the edges of necrotic foci, a few tubules may be found in prenecrotic stages, and in these, stainable fat may be seen, the significance of which will become more apparent in a consideration of pathogenesis.

Pathogenesis of the Lesion: The first detectable change in the kidney in acute choline deficiency can be demonstrated satisfactorily only when rather large numbers of rats are killed daily during the course of the experiment because of great individual variation amongst animals in the speed with which the lesion develops. In a serial experiment, animals killed on any particular day may exhibit various stages of the lesion, from normal to almost frank tubular necrosis and hemorrhage. Therefore, to say, as some authors have, that only a few animals may exhibit any particular change is readily explained by the fact that different stages are being simultaneously observed.

Our studies indicate the following sequence of events in kidneys of choline-deficient weanling rats. In susceptible animals, sometime during the first four days, stainable droplets of neutral fat appear in the cytoplasm at the bases of the epithelial cells of the proximal convoluted cortical tubules at the lobular peripheries. The cells simultaneously swell ("cloudy swelling"), expanding both outwards and inwards thereby diminishing both intertubular spaces and tubular lumena. We believe tubular swelling explains the reason for the initial enlargement of affected kidneys before vascular stasis and hemorrhage are obvious causes, and accounts for the shiny, tense capsules seen by naked eye-inspection. At this stage the kidneys often are actually paler than normal (rather than hemorrhagic) and yellow, presumably due to the tubular fat.

Following these events, hemostasis in capillaries in middle and inner zones of renal lobules with onset of tubular necrosis at peripheries rapidly ensues. Hemorrhage from rupture of distended capsular capillaries (unsupported by swollen parenchyma on all sides as in deeper cortical zones) is responsible for the grossly apparent red appearance of the renal surfaces and hence Dr. Griffith's apt term, "hemorrhagic kidneys."

Studies of intrarenal circulation at all the stages described above demonstrate ischemia of capillaries at the lobular periphery secondary to their compression by swollen, fatty tubules—the TOC-mechanism—tubular obstruction of capillaries. Obstruction of postglomerular blood flow at these sites readily explains stasis and engorgement proximally (in terms of direction of flow of blood) in capillaries of midzonal and subcapsular regions and hemorrhage at the latter site. Glomeruli are spared temporarily for the same reason because their blood flow is not yet appreciably impaired. Stainable fat in necrotic tubules is rapidly engulfed and removed by tissue and blood macrophages. Tubular fat therefore can be demonstrated only in the earlier, prenecrotic phase. It is a transient phenomenon for this reason, but nevertheless of fundamental pathogenic importance.

I should like to discuss two hypotheses which have been advanced to explain the hemorrhagic renal syndrome. The first, proposed by Dessau and Oleson (*Proc. Soc. Exper. Biol. & Med.* 64: 278, 1947) conceives that necrosis is caused by renal vasospasm; the second, posed by me later in 1948, suggests that the initiating event is that of deposition of stainable fat in proximal convoluted tubules with subsequent swelling, and initiation of TOC-induced tubular ischemia and necrosis. Both theories have in common an ischemic mechanism for the necrosis, but differ in their explanation of the cause of ischemia and its site in the renal vascular tree.

The investigations of Trueta *et al.* (*Bristol Med. Chir. J.* 65: 16, 1948) clearly establish the distribution of lesions in kidneys subject to renal vascular spasm. When spasm develops in renal vessels its site is in interlobular arteries which arise from arcuate arteries and run through the cortex between adjacent renal lobules, terminating just beneath the capsule. Spasm here is preglomerular in terms of renal blood flow. Unless one believes that renal vascular spasm can occur in capillaries, the distribution of lesions in the hemorrhagic renal syndrome cannot be explained on the basis of vasospasm because, as we have shown, lesions are clearly postglomerular in terms of renal blood flow. Second, if one accepts an interference in renal blood flow, obstruction must exist far enough along the path of renal blood flow to produce extreme stasis, congestion, and some hemorrhage in those parts of the vascular tree proximal to the site of obstruction. If renal vasospasm is the explanation, one would have to believe that it occurred at the terminal end of the capillary network just proximal to its drainage into interlobular veins in order to explain the extreme stasis and congestion proximally. We should like to emphasize that the only site of renal vasospasm thus far demonstrated occurs in interlobular arteries, and occlusion at this site could scarcely explain the extreme congestion and stasis distal to it. It is true that if spasm did occur in interlobular arterial, there would be a drop in the *vis a tergo* with the result that some stasis in renal vessels distal to the obstruction might be expected. But it would be merely a passive accumulation of red blood cells, producing a mild degree of vascular distention, surely never sufficient to distend vessels, even weak subcapsular ones, as to rupture them producing hemorrhage.

Experiments by Baxter (*Am. J. Path.* 24: 503, 1948) who used large numbers of animals, have clearly demonstrated that decapsulation partially protects kidneys of young rats against hemorrhagic renal syndrome of choline deficiency. This observation supports the TOC theory, and not that of renal vasospasm, but it is only logical to assume that ischemia caused by increased intrarenal tissue tension would be at least partially relieved by decapsulation. In Baxter's hands this fact was conclusively demonstrated. We failed to demonstrate it ourselves earlier in a smaller series of animals, but the diet employed was so severely hypolipotropic that it could have overwhelmed any partial protection afforded by renal decapsulation.

If renal vasospasm were the mechanism by which cortical necrosis developed, one would scarcely explain the immunity of glomeruli and distal convoluted tubules in early and intermediate stages in development of the lesion because to do so, one would have to presume selective sites of spasm in the renal vascular tree which would spare both these structures. If one postulated spasm at some postglomerular site, it would have to involve selectively only those capillaries supplying proximal portions of the nephron, and not those supplying distal. On the other hand, distal convoluted tubules do not develop stainable droplets of fat within their cytoplasm as do proximal, nor do the distal become swollen as do the proximal. Distal convoluted tubules occupy central portions of renal lobules, and as we have shown, necrosis is most extensive at the peripheries of the renal lobules where only proximal convoluted tubules are found. Swelling of proximal convoluted tubules here, therefore, has a more profound ischemic effect on intervening capillaries than in the center of the renal lobule where some tubules are distal and escape fatty change and swelling. After obstruction of blood flow at the peripheries of the renal lobules where blood is drained into interlobular veins, stasis and engorgement develop proximally to such a degree that glomeruli and distal convoluted tubules may eventually and much later exhibit secondary necrotic changes. Moore (*J. Path. and Bact.* 74: 171, 1957) has suggested that in terminal stages actual thrombosis of interlobular renal arteries may occur leading to lobular infarcts. Although we have not observed this phenomenon, it is a distinct possibility. But even Moore suggests that it would represent only a secondary phenomenon following extensive inflammatory reaction that develops in these kidneys with the onset of initial necrosis of proximal convoluted tubules.

As early as 1940, Goldblatt emphasized that any theory attempting to explain the pathogenesis of the hemorrhagic renal lesion must account for the paradoxic recovery and partial healing that spontaneously occurs in a small but consistently predictable percentage of the young animals, *even without any change in dietary regimen*. If one subscribes to the hypothesis of renal vasospasm, it might be postulated that hypertonicity of vessels eventually merely passes off and restores blood flow. But the TOC theory inherently provides an explanation which thereby even supports the concept. Necrosis, dissolution, and removal of cellular debris derived from the ischemic tubules at lobular peripheries would automatically relieve the pressure from without on the regional capillaries thereby permitting restoration of blood flow here and recovery in those animals which had not yet succumbed to renal failure. Obviously, the timing of events here would be so crucial that tubular necrosis and dissolution could result in the survival of only a small percentage of animals, and in actual fact, only a small number do live unless choline is restored to their diet.

Dr. Artom (*Nature* 171: 347, 1953) has shown an increase in the rate of fatty-acid oxidation of kidneys of young weanling choline-deficient rats a few hours after treatment with choline. His finding is fundamental, and may help explain the accumulation of tubular fat that we see histologically. We believe that his observations, together with ours, strongly suggest that in the elucidation of mechanisms or modes of action of lipotropic factors in preventing renal hemorrhagic syndrome, attention should be focused in the area of fatty-acid oxidation by proximal convoluted tubules. We believe that a search by physiologists

and biochemists to demonstrate some effect of dietary choline on the tone or spasm of renal vessels would not be pertinent to the problem since evidence indicating a primary role of renal vasospasm in the pathogenesis of the hemorrhagic syndrome of choline deficiency is not convincing. Nor does it explain the distribution or sites of lesions encountered. Our morphologic observations and hypothesis based on TOC do not explain the *action* of choline in protecting weanling rats against this acute lesion. We believe they indicate a pathway for further investigation of the role of choline in metabolism of fat by proximal convoluted tubules.

Dr. Olson: Helen Deane and I, at Harvard about ten years ago, sought to correlate the changes in the cytochemical structure of the kidney of the choline-deficient rat with the respiration of its kidney tissue *in vitro* (*J. Nutrition* 39: 31, 1949). At the time we did the work, the elegant sucrose flotation technic of Hogeboom for the isolation of mitochondria had not yet been proposed and we depended upon a histochemical technic (fixation in formol-calcium, postchromation, and staining with acid hematin) for evaluation of the integrity of the mitochondria. In these studies it was very clear that the changes in the mitochondria (fragmentation and even dissolution) and the changes in respiration of corresponding kidney cortex slices, which paralleled them (depression of Q_{O_2} in Krebs-phosphate medium with glucose as substrate from 20 to about 9) came late. In other words, the appearance of fat in, and swelling of, the kidney tubules, congestion of blood in the peritubular plexuses, and stasis beneath the capsule *preceded* the alteration in the structure of the mitochondria and the concomitant decline in respiration of the kidney cortex.

This was very disappointing to us because it was our hope, based largely upon the studies of Patterson and McHenry (*J. Biol. Chem.* 156: 265, 1944) already alluded to by Dr. Griffith, that the disintegration of the mitochondria, secondary to a hypothetic "choline effect" upon phospholipid synthesis in the mitochondria, might have preceded the tubular swelling and blood stasis in the surrounding vessels. Instead, we are left with ischemia as the probable cause of the mitochondrial disintegration.

Dr. Griffith (closing remarks): The remaining question is, why is the renal lesion of choline deficiency found only in the rat? I think we are dealing here with a very unusual circumstance, the combination of a great need in a very rapidly growing animal. I am intrigued by Chanutin's concept of chemical maturity in the rat, as I mentioned in my paper. There is evidence for this, also, in the studies of choline oxidase, and I am sure that if one were to make a systematic study of the tissue levels of other enzymes during this period of very rapid growth in the rat, some light might well be thrown on this phenomenon.

As far as the sex differences are concerned, I have no answer other than that there are many illustrations of variations in lipid metabolism between the two sexes.

Vascular Disease Associated with Choline Deficiency in the Rat

GEORGE F. WILGRAM, M.D., PH.D.*

ALTHOUGH fatty liver[1] and hemorrhagic renal degeneration[2] as a consequence of dietary choline deficiency in the rat have been known for many years, it was not until 1952 that Hartroft, Best and their colleagues[3] described pathologic changes in the major arterial trunks (aortas and carotid arteries) and in the coronary arteries of rats maintained up to 216 days on diets low in choline. Somewhat later these studies were extended in our laboratory to the investigation of vascular changes in acute severe choline deficiency.[4] Following this, further work was undertaken to elucidate the etiology, pathogenesis, and biochemistry of the cardiovascular lesions in choline deficiency.[5]

A thorough investigation of this problem seemed to be strongly indicated in view of the great interest in the role of lipotropic factors in nutritional disorders as well as their possible significance in the vast complex of cardiovascular pathology.

HISTOPATHOLOGY

Young choline-deficient rats may develop a number of cardiovascular lesions after three weeks in acute, or after approximately five months in long term, experiments.

A focal cardionecrosis is found preceded by the appearance of stainable lipid within the muscle fibers (Fig. 1). Polymorphonuclear leucocytes and later lymphocytes appear at the site of lipid-laden muscle fibers. Interstitial edema is frequently pronounced. Sometimes death of the animals ensues before this interstitial myocarditis can further develop into frank focal necrotic areas. If the animal survives, the inflammatory and necrotic debris is absorbed and removed, leaving focal fibrotic scarred tissue in the myocardium. No definite topographic relation between the coronary tree and these focal necrotic areas has as yet been discerned.

Stainable lipid mainly in the media of coronary arteries is noted (Fig. 2). Intra- and extracellular lipid also may be observed in the intima and the adventitia but the location of main accumulation is the media. Sometimes this stage of lipid appearance is followed by signs of necrosis in the components of the media.

The same picture as in the coronaries is observed in the aorta of choline-deficient rats except that medial necrosis is much more pronounced and much more common. This medial necrosis is then followed by calcification and the aorta is frequently totally rigid, hardened, and widened with the appearance of a bamboo stick (Fig. 3). The intima of the aorta is but rarely involved and shows on occasion only a "hyaline cap" over areas that are necrotic and calcified in the underlying media. This hyaline cap is apparently a proliferative connective tissue response to the injury of the medial structures beneath.

The elastic elements of the aorta are shriveled and broken up. The whole media looks microscopically like a condensed calcified bar. On occasion this bar-like tissue breaks, leading to a picture that has been termed "collar-button fracture" by Hartroft. The whole syndrome may therefore most favorably be com-

From Banting and Best Dept. of Medical Research, University of Toronto, Toronto, Canada.

* Lecturer, Dept. of Medical Research, University of Toronto.

Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc., New York, New York.

Supported by the Life Insurance Medical Research Fund and The Nutrition Foundation.

pared with Mönckeberg's sclerosis in human arteries (Fig. 4). There is no similarity to the process of atherosclerosis as encountered in man.

ETIOLOGY AND PATHOGENESIS

The etiology and pathogenesis of those lesions may be of little *practical* significance but it is a very important theoretic matter. It is obvious that a lack of choline initiates these lesions as they are all prevented by the addition of sufficient choline to hypolipotropic diets. The mechanisms by which these lesions are induced in choline-deficient rats are still obscure despite considerable work and effort put into this problem. The main point is whether these pathologic changes are due primarily to lack of choline or whether they are secondary to renal cortical necrosis that is nearly always present in choline-deficient animals suffering from cardiovascular disease. Bilateral renal hemorrhagic cortical necrosis is induced by severe choline deficiency and it is conceivable that the ensuing pre-uremia or uremia is responsible for the tissue break-down in the vessels. We favor this view of the nature of cardiovascular disease, i.e., as being secondary to renal damage in choline-deficient rats. Others, however, feel that lipotropic factors are primarily involved in the integrity of the cardiac tissues of the rat. Hartroft favors the opinion that choline is the essential dietary component necessary for the health of the cardiac muscle while vascular changes in the aorta and coronaries may perhaps be due to renal injury. He therefore thinks that cardiac necrosis on the one hand and vascular changes on the other, are due to different etiologic factors. This concept has some experimental support and seems to be very attractive. Kleinerman,[6] however, supports the concept that it is methionine which is the component essential for the prevention of

Fig. 1

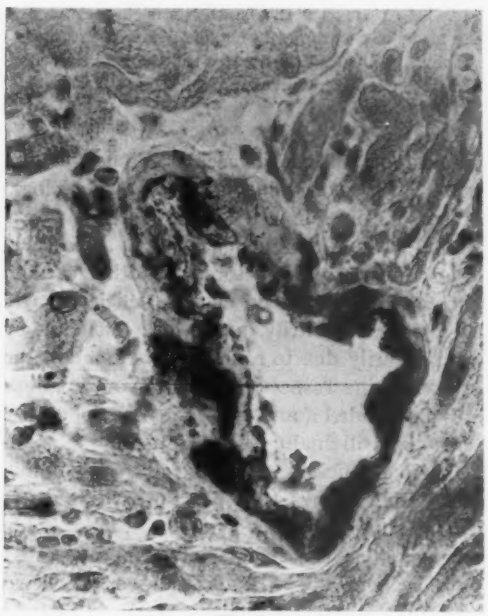


Fig. 2

Fig. 1. (High power, frozen section, Oil Red-O stain.) Fat appears as black droplets throughout the cytoarchitecture of choline-deficient myocardial fibres. The lipid accumulation within the muscle fibres is followed by edema and the appearance of polymorphonuclear leucocytes. Later on necrosis and lysis of the muscle fibres ensues. Fig. 2. (High power, frozen section, Oil Red-O stain.) Fat appears as black masses throughout the entire vessel wall in this coronary artery of a choline-deficient rat. Note, however, that the site of main lipid accumulation is the media.

Fig. 3. At autopsy the bamboo stick appearance of the aorta in a chronically choline-deficient rat is very striking. Note that the aorta is widened and that calcified rings characterize this state of aortic sclerosis.

Fig. 4. (High power, paraffin section, PAS stain.) The Mönckeberg type of medial sclerosis, seen at autopsy in the left lower photograph is demonstrated by a widening of the elastic laminae of the aorta and accumulation of cellular debris and connective tissue disintegration. The inner part of the aorta is rigidly calcified into a bar that is broken. At the site of this fracture strongly PAS-positive material is illustrated by the darkness of the area surrounding both ends of the fractured bar. The epithelium overlying this site of necrosis shows signs of cellular proliferation as manifestation of the irritation by the underlying injury of the aortic media.

break-down of cardiovascular tissues in the rat. Since methionine is one of the biologic precursors of choline it is very difficult to exclude *some* methionine deficiency in a diet that is low in lipotropic factors. Whether cardiac and vascular lesions in choline deficiency are, therefore, secondarily due to lack of choline or are primarily due to a deficiency of choline or of methionine, respectively, can, in our opinion, only be decided if and when the mode of action of choline will finally be elucidated.

Choline-deficient animals always exhibit blood lipid values that are lower than their choline-supplemented controls. This holds true whether the blood lipids are expressed physicochemically as lipoproteins or biochemically as cholesterol, phospholipids or neutral fat.[7,8,9] In contrast to the low blood values are the levels of liver lipids in choline-deficient animals which, of course, are always elevated as compared with their choline-supplemented controls. In atherosclerotic humans and in experimental animals the blood lipid values are either elevated or within the normal range, but they are only rarely decreased. This difference in biochemical pattern also separates cardiovascular disease in choline-deficient rats from atherosclerotic processes in man.

CLINICAL APPLICATIONS

The interest of an audience such as the one assembled for this symposium is often focused on possible clinical applications. Both lipotropic phenomena and atherosclerosis command great attention these days. We feel obliged, however, to come to the conclusion that cardiovascular disease as observed in choline-deficient rats has no direct clinical bearing on the vascular lesions which are en-

countered most frequently in North America. The reasons are as follows:

(1) Pathologically, the lesions resemble Mönckeberg's sclerosis and not those of atherosclerosis. The sequelae of Mönckeberg's sclerosis are not nearly as deleterious to health as those of atherosclerosis.

(2) The biochemical lipid patterns in choline deficiency and in atherosclerosis are altered in opposite directions.

(3) A relationship between choline deficiency and human Mönckeberg's disease is at present not established because patients having Mönckeberg's vascular sclerosis do not seem to suffer from dietary lack of choline here in North America. Whether there is an intrinsic defect of choline metabolism in those patients remains to be investigated.

(4) A clinical application of the findings in choline-deficient rats to areas where malnutrition and kwashiorkor are common is perhaps possible. But here again the complex problem of protein malnutrition is hard to separate from an inadequate supply of choline in the diet. We have pointed out before that a choline-deficient diet is frequently border-line deficient in methionine as well, and therefore, choline and protein deficiencies are not easy to differentiate.

(5) So far, no species other than the rat has been observed to develop this type of vascular disorder in choline deficiency. This would lead one to assume that there is a specific reason for this susceptibility of the rat which does not occur in other species.

To make clinical applications of an experimental finding requires that several species including man should exhibit similar, or at least comparable results. As the vascular lesions in choline-deficient rats seem to be confined to this one species, it seems reasonable to be cautious about making any extensive clinical comparisons or applications.

Recently Williams[10] has described cardiac necrosis in choline-deficient mice. But here, too, as Dr. Williams points out,[11] protein deficiency may be the etiologic factor because even choline-supplemented controls may exhibit cardiac necrosis on those protein-poor diets.

THEORETICAL IMPLICATIONS

Although we feel that we cannot subscribe to a direct clinical application of the findings in choline deficiency we still believe that this type of work is of great importance. Choline is certainly of significance in the metabolism of fat in the liver and its release into the blood. The whole problem of lipid transport is currently being reinvestigated in many laboratories and choline seems to play some role in these complicated processes. A severe lack of dietary choline leads in rats to a breakdown of cardiovascular tissues and it would be of great importance to know by what mechanisms the integrity of the cardiovascular tissue structure is maintained.

While all agree that choline is a dietary essential for man as well as for animals it appears that normal mixed diets supply adequate amounts of choline or its precursors. The therapeutic use of *extra* dietary choline does not seem to be warranted on physiologic grounds. It is obvious, however, that a broad research program on the mode of action of choline will continue to make important contributions in many scientific fields as it has in the past.

REFERENCES

1. Best, C. H. and Huntsman, M. E.: The effects of the components of lecithine upon deposition of fat in the liver. *J. Physiol.* 75: 405, 1932.
2. Griffith, W. H. and Wade, N. J.: Choline metabolism: The occurrence and prevention of haemorrhagic degeneration in young rats on a low choline diet. *J. Biol. Chem.* 131: 567, 1939.
3. Hartroft, W. S., Ridout, J. H., Sellers, E. A., and Best, C. H.: Atheromatous changes in aorta, carotid and coronary arteries of choline deficient rats. *Proc. Soc. Exper. Biol. & Med.* 81: 384, 1952.
4. Wilgram, G. F., Hartroft, W. S., and Best, C. H.: Dietary choline and the maintenance of the cardiovascular system in rats. *Brit. M. J.* 2: 1, 1954.
5. Wilgram, G. F. and Hartroft, W. S.: Pathogenesis of fatty and sclerotic lesions in the cardiovascular system of choline deficient rats. *Brit. J. Exper. Path.* 36: 298, 1955.
6. Kleinerman, J.: Effects of ethionine and high fat diets, with and without choline supplementation on the production of aortic sclerosis in rats. *Fed. Proc.* 16: 362, 1957.
7. Wilgram, G. F., Lewis, L. A., and Blumenstein,

J.: Lipoproteins in choline deficiency. *Circulation Res.* 3: 549, 1955.

8. WILGRAM, G. F., LEWIS, L. A., and BEST, C. H.: Effect of choline and cholesterol on lipoprotein patterns of rats. *Circulation Res.* 5: 111, 1957.
9. OLSON, R. E.: Effect of dietary fat, protein and choline upon serum lipids and lipoprotein of the rat. *Fed. Proc.* 16: 395, 1957.
10. WILLIAMS, W. L. and ARONSOHN, R. B.: Cardiac and hepatic lesions in mice fed yeast protein diets. *Yale J. Biol. & Med.* 28: 515, 1956.
11. WILLIAMS, W. L.: Dietary fat and the pattern of hepatic liposis in choline deficient mice. *Fed. Proc.* 16: 377, 1957.

DISCUSSION

Dr. Gerald P. Rodnan (University of Pittsburgh, Pittsburgh, Pa.): So impressive have been the findings shown to us by Dr. Wilgram that for this reason alone I would be tempted to disagree with him about the possible clinical significance of these vascular lesions. To begin with, they are strikingly similar to those of so-called Mönckeberg senile sclerosis of man. Mönckeberg sclerosis is most apparent in the medium sized arteries, in such muscular arteries as the radial and femoral. It has been pointed out that the rat's aorta bears a closer resemblance to our medium sized arteries than to our aorta because, like the medium sized arteries, the rat aorta does not have vascularization of the media. The vasa vasorum penetrate no further than the outermost part of the adventitia.

It is true that in clinical material we do not observe the accumulation of lipids shown so beautifully in the sudan stains of Dr. Wilgram's material. Of course, we are dealing in the case of the human with a disease change which has evolved over a period of decades. We know very little about the earliest lesion of Mönckeberg's sclerosis. There is a degeneration of myoelastic fibers, but how this comes about we are quite unsure.

As far as the pathogenesis of these experimental lesions is concerned, a number of suggestions have been made. In the first communication on the subject, it was conjectured that the medial lesion of the aorta was secondary to damage of the intima. As a matter of fact, the title of the original paper was to the effect that the authors had produced atheromatous lesions in the intima of the vessel (*Proc. Soc. Exper. Biol. & Med.* 81: 384, 1952).

I agree with Dr. Wilgram that the intimal lesions are rather less striking, in the aorta certainly, than are the medial lesions. This is not hard to understand when one appreciates the fact that the rat aorta has a very narrow intima indeed, consisting only of a single layer of endothelial cells.

With regard to renal involvement as a basis for the cardiac and aortic findings, the evidence appears far from complete. Certain lesions have been seen in the myocardium of experimental animals and with renal insufficiency the so-called myocytolysis described recently by Schlesinger (*Am. J. Path.* 31: 443, 1955). These lesions, however, are not characteristically necrotic in character. There is none of the accumulation of inflammatory cells which we have seen in the slides of Dr. Wilgram's material. I would rather subscribe to the idea that these changes may be a result of choline deficiency itself. I wonder whether factors which protect in experimental choline deficiency, such as betaine and vitamin B_{12}, would also protect against these myocardial lesions, and whether such agents as cystine and choline antagonists which can produce fatty changes in the liver might enhance these lesions.

As far as aortic disease in general is concerned, there is one other lesion in the media of the rat aorta which is of great interest. A form of medial degeneration, frequently associated with aneurysm, has been produced experimentally in the rat by the administration of beta-aminopropionitrile. Given to young animals there results a deficiency of intact elastic laminae, fibroblastic proliferation, and in later stages a decrease in periodic acid-Schiff-positive material (*Arch. Path.* 64: 434, 1957). At times there is also involvement of the cardiac valves. I wonder if Dr. Wilgram has noted any alterations in the valves in his experimental animals.

Dr. Hartroft: I would make a plea that the term cardiovascular not be used too readily, lumping all these lesions together. I know we frequently say "cardiovascular," but here, at any rate for a while, let us keep the cardiac lesion separate from the vascular lesion and not speak about the aortic changes and cardiac necrosis as being the same thing until we learn more about them.

I think the evidence is pretty clear that cardiac lipoidosis and cardiac necrosis are not secondary to renal lesion because the original work which stimulated this work of Dr. Wilgram's and mine was that of Stetten, *et al.* (*J. Nutrition* 29: 171, 1945) who showed that feeding young weanling rats a choline-deficient diet containing 40 per cent of ethyl laurate killed them in three or four days. They died of myocardial necrosis. Stetten, *et al.* did not study the early stage of the precursor of necrosis, the lipoidosis. We were able to confirm them and study this early stage of cardiac lipoidosis. Dr. Stetten's rats did not develop renal lesions and Dr. Wilgram and I did not observe renal lesions in our rats either (*Brit. M. J.* 2: 1, 1954).

Secondly, I think we have a counterpart for this cardiac lesion in human pathology. In certain types of alcoholics, a very flabby necrotic, fatty heart may be encountered, mostly in beer drinkers. These lesions develop in acute alcoholics who have fatty livers. Perhaps the fatty necrotic heart in the rat is a morphologic counterpart of that lesion.

The concept of whether a deficiency of methionine or of choline is the important factor in the production of the vascular lesion is something which will be resolved only by further experimentation.

Dr. Lathem: Do these animals get hypertension?

Dr. Wilgram (closing remarks): In answer to Dr. Rodnan's comments I would like to say that when I used the term Mönckeberg's disease I used it with some reservations. I am quite aware that different interpretations may be ascribed to those different lesions. It is a matter of preference and one man likes to describe those experimentally produced lesions as cystic medial (Erdheim) necrosis and others prefer to call them Mönckeberg's medical sclerosis. Again I would like to point out that I am making these comparisons in terms of the anatomy of the rat realizing that there are differences between the anatomy of the rat and the anatomy of man. Certain anatomic lesions seen in humans just cannot be found in other species! Certain features however may be seen in both species. On the basis of those common features I have chosen, perhaps frivolously, to compare those lesions with the Mönckeberg lesion in man.

Dr. Rodnan: I agree with you that there is a striking resemblance pathologically between those lesions and Mönckeberg's sclerosis in man. The circling of the vessel with calcium and many of the other findings are quite identical with what is found in the human material.

Dr. Wilgram: Coming to the second point, the occurrence of necrosis in the myocardium, I should like to say that myocardial necrosis as a consequence of kidney damage may be observed under a variety of conditions. Time does not permit my going into detail, but I could give you five different references to kidney lesions produced by different experimental means with ensuing cardiac lesions, not identical, but similar to the ones observed here.

The third question concerned involvement of cardiac valves. I have as yet, not observed lesions in the valves.

The effect of ethyl laurate upon the myocardium of the rat mentioned by Dr. Hartroft is a problem which should be reinvestigated. It is true that in those early days kidney damage was not always observed. However, Dr. Hartroft, ethyl laurate is a highly toxic substance. One out of five of our controls (*Brit. M. J.* 21: 1, 1954) showed cardiac lesions, i.e., the triglyceride of lauric acid produced some cardiac necrosis in choline-supplemented controls. This is why at the present time I am inclined to pay less attention to those early experiments.

However, I would like to re-emphasize the fact that the whole question of pathogenesis and etiology is wide open, and I cannot insist that my view is right. I just mention it for the sake of discussion. Maybe I am wrong. The future will show.

Concerning hypertension, I believe Dr. Hartroft found some years ago that in the active stage of the development of hemorrhagic kidneys hypertension develops, but if they stay on a choline-deficient diet the hypertension disappears.

Dr. Hartroft: The rats do not develop hypertension during the acute stage, only during the recovery stage.

Fatty Liver in Man and the Role of Lipotropic Factors

GEORGE J. GABUZDA, M.D.*

BASIC INVESTIGATIONS of the type discussed in this symposium undoubtedly have influenced the thinking of clinicians regarding the pathogenesis and treatment of diseases of the liver. Investigations relating nutritional factors to the production of hepatic lesions in animals[1–7] were followed by clinical reports attesting to the efficacy of nutritious diets in the treatment of patients with liver disease.[8,9] The factors in the diet that account for its reported effectiveness have not been defined. Thus, in spite of the great number of animal and *in vitro* experiments that have been done to delineate better the nutritional factors responsible for the functional and histologic integrity of the liver,[10] there is no conclusive evidence that any of these factors are operative in clinical situations. These factors are incriminated by the analogy of clinical problems to the basic animal experimental situations, and not by critical data. The evidence from animal experiments that relates nutritional factors to hepatic lesions is by this time so extensive and, in some areas at least, so conclusive, that it would seem probable that some of these findings apply to liver disease in man. The paucity of conclusive data relating specific nutritional factors to liver lesions in man is related in part to the difficulties encountered in the design and interpretation of clinical studies. These points will be further illustrated herein.

From the Department of Medicine, Western Reserve University at Cleveland City Hospital, Cleveland, Ohio.

* Associate Professor of Medicine, Western Reserve University School of Medicine, Cleveland, Ohio.

Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc., New York, New York.

FAT METABOLISM

A schematic diagram of the role of the liver in the metabolism of fat is shown in Figure 1. The liver through its function of bile secretion influences the absorption of fat and fat-soluble substances from the gastrointestinal tract. The liver also plays an important role in the intermediary metabolism of fat, since it is involved in the synthesis, storage, distribution, and oxidation of lipids in the body. Which of these functions predominates depends upon the metabolic circumstances existing at any given time. Likewise, the quantity of fat contained in or passing through the liver in a given time is related to such factors as the ingestion of or abstinence from food, the kind of food consumed, hormonal influences, etc. Liver fat content might then be increased as a result of any alteration that increases the quantity of fat presented to the liver, that limits the transport of fat from the liver, that limits the oxidation of fat in the liver, or possibly that enhances the synthesis of fat in the liver.

The following are illustrative: If excess calories are consumed, as in obesity, dietary intake of fat is increased and an increased synthesis of fat from carbohydrate occurs. These processes may be reflected by an increased liver fat content.[11,12] Contrariwise, with caloric deficit, there is an increased mobilization of fat from depots to the liver where it can be prepared for oxidation to provide energy for tissues. This increased mobilization of depot fat, possibly resulting from the action of pituitary hormones,[13–17] may result in an increased liver fat content during the early phases of starvation. As starvation proceeds and depot fat diminishes until it is essentially unavailable, the liver no longer contains excess fat. Increased quantities of fat occur in the livers of

patients with diabetes who are not adequately controlled.[18–21] In the absence of adequate insulin, there is an inability to utilize carbohydrate adequately. Fat is mobilized from the depots and the synthesis of fat from carbohydrate is diminished. The increased metabolism of fat in the uncontrolled diabetic is reflected by an increase in liver fat content, the fat being on the way to conversion to ketone bodies which can then be oxidized in the liver or other tissues. With choline deficiency there

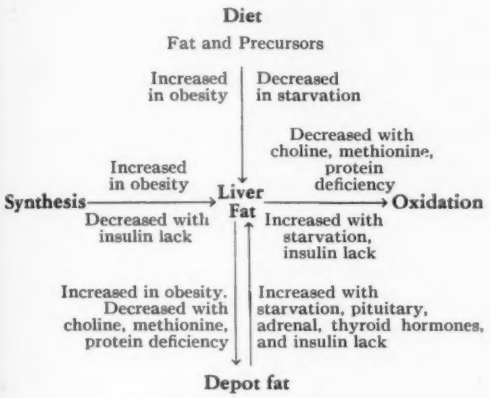


Fig. 1. The liver in fat metabolism.

is decreased transport of fat from the liver to fat depots and a decreased oxidation of fat in the liver.[22,23] As consequences, the liver fat content is increased in animals under conditions of dietary choline deficiency.

Alterations in any of the mechanisms concerned with the metabolism of fat may result in an increased content of liver fat. Dietary deficiencies are not necessary prerequisites for this, but it may also result from dietary excesses or disturbances in intermediary metabolism.

Some etiologic factors associated with increased fat in the liver are listed in Table I. This tabulation indicates that a number of clinical conditions may be associated with an increased liver fat content in the absence of alcoholism. This point is further illustrated by the data presented in Table II. These data from the report of Thannhauser and Reinstein[24] indicate that liver fat content may be increased in patients with various diseases. Although marked increases are admittedly most consistently seen in this country in patients with alcoholic liver disease, other patients, e.g., the patient with subacute bacterial endocarditis and one of the patients with celiac disease (Table II), may have quite large quantities of fat in the liver in the absence of alcoholism.

TABLE I

Some Etiologic Factors in Fatty Liver

- A. Nutritional
  - 1. Starvation
  - 2. Low Protein Diet
  - 3. High Fat Diet
  - 4. High CHO Diet
  - 5. Choline Deficiency
  - 6. Methionine Deficiency
- B. Hormonal-metabolic
  - 1. Pituitary
  - 2. Adrenal
  - 3. Thyroid
  - 4. Insulin
  - 5. Sex Hormones
- C. Toxic
  - 1. Chemical (CCl_4, $CHCl_3$, P)
  - 2. Bacterial
  - 3. Anoxic
    - (a) Anemia
    - (b) Congestion
- D. Combined factors
  - 1. Combinations of above

THE LIVER IN ALCOHOLISM

Many of the etiologic factors listed in Table I may be involved in the production of fatty liver in the alcoholic. For instance, the alcoholic who presents with a fatty liver may have undergone a period of starvation. He may have been consuming a diet low in protein or a high-caloric diet in which most of the calories are

TABLE II

Liver Fat in Various Diseases*

	Neutral fat g/100 g
Normal	1.4–4.0
Subac. bact. endocarditis (1)	35.1
Pneumonia (1)	6.7
Tuberculosis (1)	8.4
Celiac disease (2)	5.6, 52.0
Alcoholic cirrhosis (6)	38.2, 38.0, 56.5 65.5, 52.9, 19.8

* Modified from Thannhauser, S. J. and Reinstein, H., *Arch. Path.* 33: 646, 1942.[24]

derived from alcohol. He may therefore, have been subsisting on rations providing for a discrepancy between total caloric intake and the intake of protein or other essential nutrients.[25] Evidences of specific nutritional deficiency disease may be exhibited. The alcoholic patients may have sustained physical injury or infection, or as a result of withdrawal may be suffering with delerium tremens. These associated phenomena may well serve as stimuli for activating the pituitary-adrenal axis to elaborate hormones which are known to influence the content of liver fat. In addition, alcohol *per se* may be a hepatotoxin.

Upon what then is the etiology of fatty liver in the alcoholic based? It is indeed difficult to decide. The convincing evidence from animal experiments that incriminates deficiency of such lipotropic factors as choline and methionine and the supporting clinical evidence that many alcoholics exist on nutritionally deficient rations cannot be ignored. Yet it is not possible to state dogmatically that deficiency of one factor or another accounts for the development of fatty liver in the alcoholic. In fact, alcoholics who present with fatty livers do not always show clinical evidence of undernutrition or specific vitamin deficiency diseases. Indeed, some of these patients appear "well nourished," and others are obese. It is possible that multiple factors are involved and that the specific factors involved vary from individual to individual.

Fatty liver is classically associated with alcoholism in this country. Whether justified or not, the clinician is prone to consider fatty liver in the alcoholic as the pathologic lesion of primary concern, and in the nonalcoholic patient to pay little attention to it Patients with metabolic, gastrointestinal, infectious, neoplastic diseases, etc., may have fatty livers in the absence of alcoholism. In these situations a fatty liver is considered a secondary and incidental finding apparently of little consequence. It is presumed that with adequate treatment of the primary disease process, without particular emphasis on the treatment of the associated liver lesions, these will reverse; apparently they do. On the other hand, in the case of fatty liver associated with alcoholism, the clinician's primary concern is directed toward the increased liver fat content. The possible association of fatty liver to the subsequent development in alcoholic patients of hepatic fibrosis with all the stigmata and consequences of chronic hepatic disease may account for this, although the relation between fat and subsequent fibrosis is still the subject of debate.[26–30]

CLINICAL FEATURES

In any event, the significance of increased liver fat content is difficult to assess with regard to both its immediate and long-term consequences. Patients, whether they be alcoholic or not, may have fatty livers and present no symptoms or signs relating to the liver. In these instances the diagnosis can be made only by biopsy of the liver. This is exemplified by the high incidence of fatty liver found by biopsy in alcoholic patients hospitalized with delerium tremens,[31] many of whom do not have clinical evidence of hepatic disease. Other patients who enter the hospital with fatty livers, with or without overt clinical evidences of liver disease, may die suddenly and unexpectedly. In these cases the liver is usually loaded with fat, and this may be the predominant finding at necropsy. The exact cause of death in these instances is not usually known. In other patients hepatomegaly may be the only clinical sign suggesting the presence of fatty liver. Between these extremes are alcoholic patients who enter the hospital with varying degrees of clinical and laboratory evidence of liver disease. The patients are likely to display tender hepatomegaly and jaundice; however, ascites, edema, spider angiomata, and splenomegaly are not common findings. There is almost always increased bromosulphalein retention, and frequently hyperbilirubinemia and an increased excretion of urobilinogen in the urine. Serum albumin and globulin concentrations, prothrombin time, thymol turbidity, cephalin flocculation, and alkaline phosphatase are less frequently abnormal, but any or all of these tests of liver functions may deviate from the normal.

These patients have a strong tendency to become well following hospitalization, alcohol

withdrawal, and the provision of even modest quantities of food.[32–36] Subjective improvement in appetite and decrease in liver tenderness may be apparent during the first few days in the hospital; abnormal tests of liver function may revert to normal in a week or two, and liver fat content may become normal in four to six weeks. Although most of these patients undergo favorable courses as outlined, liver fat persists in some in whom it is associated with evidence of cellular necrosis, fibrosis, and increasing evidence of liver damage. This may end in several weeks with death due to liver failure, or undergo a process of repair and regeneration. Evidence of liver dysfunction may persist and in the future result in hepatic failure.

The clinical pictures associated with fatty liver lesions are obviously quite varied. The reasons for this variation are not apparent. There is not always a close correlation between the degree of involvement of the liver with fat and the clinical findings which the patient presents. There is need for more adequate information concerning the significance of abnormal quantities of fat in the liver. The extent and duration of fatty involvement of the liver may be determining factors, but this is not established. Perhaps the consequences depend upon such factors as the mechanism whereby liver fat content is altered, the rate at which fat accumulates or fails to be mobilized, the source or the type of fat concerned, in addition to its morphologic distribution. These possibilities are deserving of more attention. For instance, it may be that certain biochemical situations produce fatty livers which are easily reversible, while others are associated with less favorable outcomes.

LIVER FUNCTION

What influence large quantities of fat within a liver cell have upon the functional ability of that cell is another question that remains unanswered. As noted above, liver cells may be greatly distended with fat and yet reveal no evidence of malfunction as judged by the usual clinical and laboratory tests which are admittedly gross. That distention of the liver with fat may interfere with function on a mechanical basis must also be considered.[5] Liver tenderness may be a manifestation of capsular distention. Bromsulfalein retention and hyperbilirubinemia may be more reflections of circulatory deficiency than abnormalities in cell function. In addition, it is difficult both clinically and by tests of liver functions to distinguish alterations that occur secondary to increases in fat content from those resulting from necrosis of liver cells, since necrosis in varying degree frequently accompanies the presence of fat, although the exact relation between these two processes is not clear.

It is apparent that the problem of investigating fatty liver in man is not an easy one. First, multiple factors may be involved in its causation. Second, in contrast to controlled experimentation with laboratory animals, the clinical investigator of necessity begins with a liver lesion which is in existence, which developed under uncontrolled circumstances, and about whose background relatively little is known or can be ascertained. For instance, historical facts such as whether a patient drank alcohol excessively while consuming an inadequate diet up to the time of coming to the hospital, or ceased drinking and began to eat prior to hospitalization may influence the clinical picture, as well as the ultimate course. It is possible that any dietary deficiency which might have existed may have been partially corrected by the consumption of food or a change in the pattern of alcohol and food intakes prior to hospitalization. Although such historical features are undoubtedly important, they are not easily established.

Several approaches have been applied to the study of fatty liver in man. Some recent clinical investigations have been selected for presentation to exemplify some of these experimental approaches.

CLINICAL INVESTIGATIONS

Radioactive phosphorus has been used to determine the rate of turnover of phospholipid in normal subjects and in patients with liver disease.[37–44] The demonstration of an increased phospholipid turnover after choline administration to patients with fatty livers would provide evidence for choline deficiency in these patients. Significant increases in

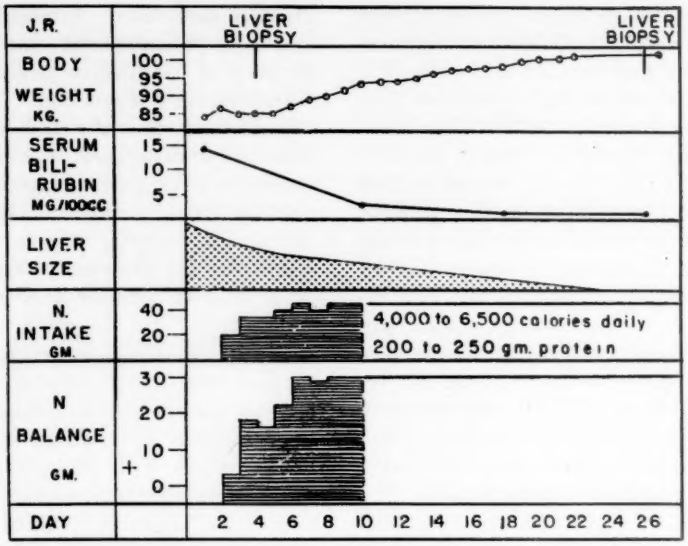


Fig. 2. Clinical course of patient J. R. The biopsies are shown in Figure 3. (From: Chalmers, T. C. and Davidson, C. S.: *New England J. Med.* 240: 449, 1949. Reproduced with the permission of the authors and publishers.)

phospholipid synthesis were noted after choline was given orally to some patients with hepatic cirrhosis. The most marked increases were noted in patients with fatty infiltration of the liver. However, some of these patients demonstrated an increased rate of phospholipid turnover after choline, but did not demonstrate decreases in liver fat content as observed in hepatic tissue obtained by biopsy. An additional patient with constructive pericarditis, a situation not obviously related to choline deficiency, also demonstrated an increased phospholipid turnover after choline administration. The apparent discrepancies detract from the

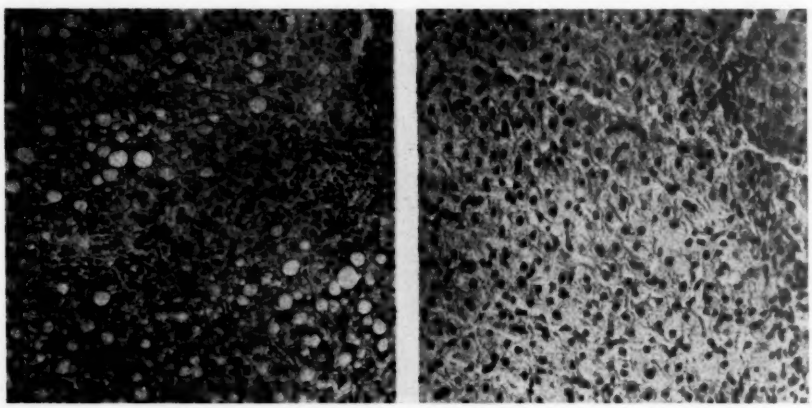


Fig. 3. Patient J. R. Liver biopsy on left done shortly after hospital admission shows moderate fatty metamorphosis and cellular infiltration. The biopsy on right after about three weeks of the nutritious diet shows essentially normal liver. (From: Chalmers, T. C. and Davidson, C. S.: *New England J. Med.* 240: 449, 1949. Reproduced with the permission of the authors and publishers.)

conclusiveness of these data, but should not discourage further investigations utilizing this experimental approach.

Other studies deal with the metabolism of orally administered choline in normal individuals and in patients with liver disease.[45–47] About two-thirds of choline administered orally to normal adult man is recovered in the urine as trimethylamines which are formed from choline in the gastrointestinal tract by the action of micro-organisms. Under similar conditions, patients with liver disease excreted less trimethylamine in the urine than the normal subjects, and the excretion was somewhat delayed. This difference suggests that patients with liver disease absorb and retain greater amounts of orally administered choline than normal subjects. A choline deficiency in patients with liver disease is implied, but here again the evidence is indirect and inconclusive. Other interpretations of these findings are also feasible. For instance, the gastrointestinal flora may differ in patients with liver disease compared to normal subjects, or the presence of liver disease *per se* may influence the way in which choline is metabolized.

Methionine metabolism in patients with liver disease has been the subject of several investigations.[48–52] In some patients these studies demonstrated increased plasma methionine levels, increased urinary excretions of this amino acid, and a limited ability to remove intravenously administered methionine from the blood. Although these findings indicate abnormalities in the metabolism of methionine, they provide no evidence for methionine deficiency. If alterations in the intermediary metabolism of methionine are related to the production of fatty liver, the relationship is not clear on the basis of available data.

Based on the hypothesis that a nutritious diet is of therapeutic value for patients with fatty liver lesions, other recent investigations have been directed toward determining the factor(s) contained in or accompanying the administration of the diet that accounts for its beneficial effect. Some of these are presented here briefly.

The therapeutic efficacy of a nutritious diet in the absence of dietary supplements, the clinical course, and the reversibility of a fatty liver lesion is demonstrated by patient J. R. (Fig. 2).[53] This 35-year-old longshoreman was a typical "spree" drinker who consumed large quantities of alcohol and ingested very little food during a period of several weeks prior to hospitalization. He was severely jaundiced, and had an enlarged and tender liver. Upon hospitalization he voluntarily consumed 4,000 to 6,500 calories daily including 200 to 250 g of protein. He gained 15 kg body weight. The serum bilirubin concentration returned promptly toward and then to normal. Liver size decreased and tenderness promptly subsided. After three weeks in the hospital this patient had no clinical or laboratory stigmata of liver disease. A liver biopsy done several days after admittance to the hospital showed a moderate fatty change and cellular infiltration. The histologic appearance of a second biopsy obtained after about $3^1/_2$ weeks of hospitalization during which the patient ate well, rested, and abstained from alcohol was essentially normal (Fig. 3).

Improvement in other patients with liver disease has been observed following the institution of a quite different dietary regimen.[54] Four patients with "active cirrhosis" were provided with a diet essentially devoid of protein but providing adequate calories. The sole source of nitrogen was provided by the intravenous infusion of a protein hydrolysate daily for two to four weeks. The basal diet contained 30 to 100 mg of choline, and the infused amino acids contained 2 to 4 g of methionine, depending upon the total quantity of amino acids given. A multiple vitamin capsule was also given to each patient daily. The four patients studied improved as evidenced by decreases in serum bilirubin concentration and urine bile, and decrease in liver size in two of the patients. The findings in one of these patients (W. C.) are presented in Fig. 4. Liver biopsies were obtained from this patient prior to the study and again after 20 days. Improvement in the histologic appearance of the liver was apparent in the second biopsy specimen. One of the patients in whom improvement occurred received insufficient amino acids to maintain a positive balance of nitrogen during

a two-week period. This latter observation suggested that improvement in liver function in man might occur even if nitrogen balance is not maintained. It suggested the feasibility of studies in which the intake of protein (and lipotropic substances) might be limited. This would allow for a better definition of the minimal therapeutic regimen required in patients with fatty livers, and possibly permit for a critical evaluation of specific agents of potential therapeutic value.

tion the patient consumed a calorically adequate but protein-free diet; there was a negative nitrogen balance, but most laboratory tests of liver functions showed considerable improvement. The serum bilirubin decreased from 9.5 mg to about 2 mg/100 ml, thymol turbidity decreased, urinary urobilinogen and bile decreased to normal. Clinically, the edge of the liver decreased from 9 cm to 4 cm below the right costal margin, hepatic tenderness subsided in a few days, and the patient felt

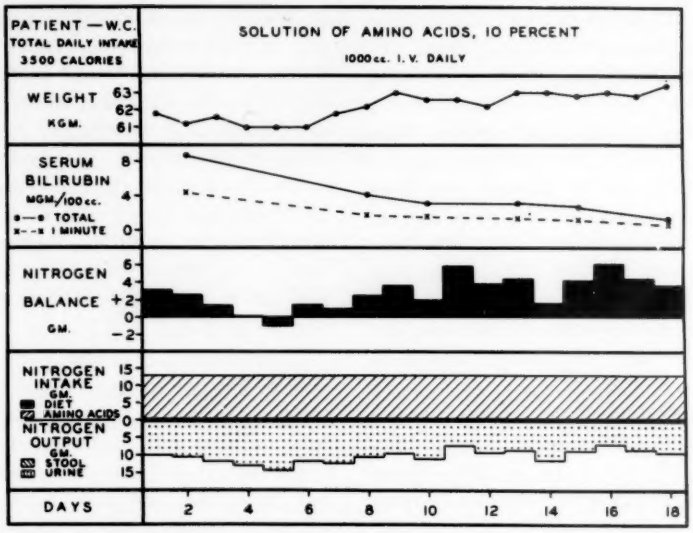


Fig. 4. Improvement of active liver cirrhosis in a patient maintained with amino acids intravenously as the source of protein and lipotropic substances. (From: Eckhardt, R. D., Faloon, W. W., and Davidson, C. S.: *J. Clin. Investigation* 28: 603, 1949. Reproduced with permission of the authors and publishers.)

Accordingly, three alcoholic patients with fatty cirrhosis of the liver were studied under conditions of protein starvation followed by protein feeding.[33] Serial observations were made on the clinical course, laboratory tests of liver functions, histologic study, and nitrogen balance. The basal control diets consisted of fat and carbohydrate (2,700 to 3,000 calories daily), a multiple vitamin capsule daily, and were essentially devoid of protein. The plan of study and the data on one of the patients (M. P.) are shown in Fig. 5.

During the initial 11-day period of observa-

well, demonstrated increased mental and physical activity, and experienced increasing improvement in appetite. With the subsequent provision of a nutritious diet, including adequate protein, a positive nitrogen balance occurred, but further alterations in the tests of liver function were minor compared to those seen during the initial period of protein deprivation. In fact, the patient's course during the period of protein deprivation was so favorable that a beneficial influence of lipotropic substances would have been difficult to detect had they been given. The improvement noted both

clinically and by laboratory tests was not, however, reflected in the histologic appearance of the liver (Fig. 6). The liver biopsy obtained initially contained a large amount of fat which was not significantly altered after 10 days of the protein-free diet. Liver fat did decrease after 19 days of the nutritious diet. A discrepancy between the clinical and laboratory criteria of improvement and the histologic appearance of the liver is apparent. The restoration of the histologic appearance toward normal depended upon the provision of a nutritious diet containing protein, while improvement as judged by clinical and laboratory criteria occurred even when protein was not fed. The study of this patient, and of two additional similar patients included in this report of Eckhardt and coworkers,[33] points up the necessity for adequate control observations and for evaluating patients by liver biopsy, as well as by clinical criteria in studies designed to test the therapeutic value of specific agents in patients with fatty livers.

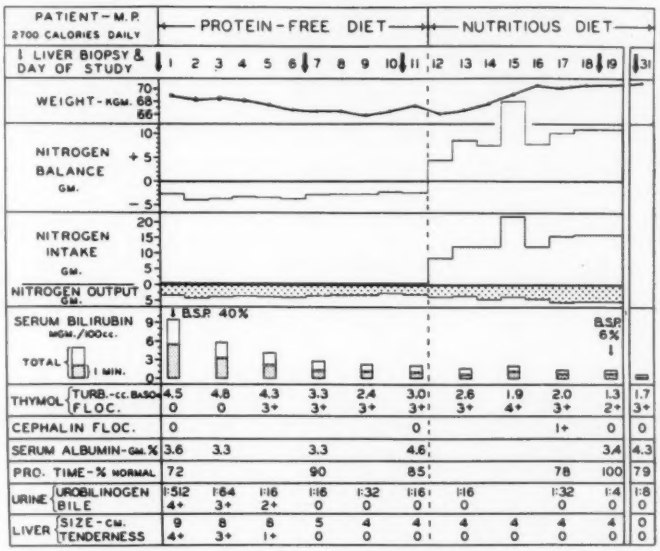


Fig. 5. Clinical course, nitrogen balance, and laboratory tests of liver function in a patient during a period of protein starvation followed by protein feeding. (From: Eckhardt, R. D. *et al.*: *J. Clin. Investigation* 29: 227, 1950. Reproduced with the permission of the authors and publishers.)

Several additional morphologic findings reported by these workers[33] are of interest. Special staining technics were employed to estimate the protein (pentose nucleic acid) and glycogen contents of the liver specimens obtained by serial biopsy during the periods of protein starvation and of protein feeding. Findings were similar for the three patients studied. Protein content was low initially, decreased further during periods of protein starvation, and increased progressively following the ingestion of the well-balanced nutritious diets (Fig. 7). Initially, a moderate quantity of glycogen was present. This increased during the administration of the protein-deficient but calorically adequate diet. Although these data are not quantitative, and cannot be interpreted too strictly, several points are worthy of mention. For example, the depletion of liver cell protein noted while patients consumed rations grossly inadequate in protein did not result in further deterioration in the patients' clinical status or laboratory tests of liver functions. This finding also suggests that under the condition of this study mobilization of protein from

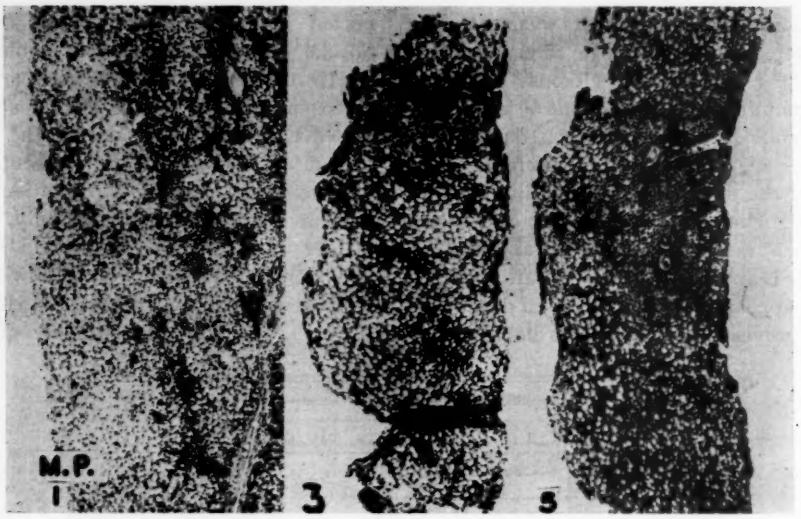


Fig. 6. The liver fat is seen as non-staining vacuoles in the cell cytoplasm. The liver of M. P. initially contained large amounts of fat. This remained unchanged after the protein-free diet was given for ten days (Biopsy No. 3), and decreased moderately after the nutritious diet was provided for 19 days (Biopsy No. 5). ×30. (From: Eckhardt, R. D. *et al.: J. Clin. Investigation* 29: 227, 1950. Reproduced with the permission of the authors and publishers.)

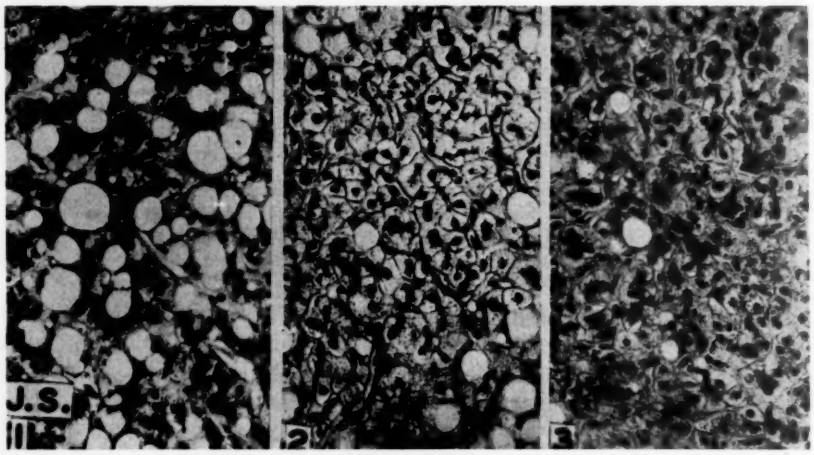


Fig. 7. Pentose nucleic acid appears as dark-staining granules in the cytoplasm of the liver cells. These granules in the initial liver biopsy of J. S. were clustered in moderate amounts in some cells, but were deficient in others (Biopsy No. 1). After receiving the diet deficient in protein for seven days, pentose nucleic acid was depleted in all cells (Biopsy No. 2). After six days of increased protein feeding, there was a return of the granules which were uniformly distributed throughout the cell cytoplasm (Biopsy No. 3). ×160. (From: Eckhardt, R. D. *et al.: J. Clin. Investigation* 29: 227, 1950. Reproduced with the permission of the authors and publishers.)

other tissues to the liver could not be depended upon for repletion of liver cell protein, nor could the improvement noted be accounted for on the basis of this mechanism. The increased quantities of fat and decreasing quantities of protein in the liver during the short period of protein starvation did not prevent the storage of glycogen.

Subsequent studies were planned to outline further the role of diet in the treatment of patients with fatty liver disease.[55] The conditions of dietary control for these patients were still more rigid than for those cited above. Three alcoholic patients with fatty livers were observed initially for 8 to 10 days during which they ingested purified diets consisting of glucose solution (1,600 cal) containing minerals, and vitamin supplements. These patients were then given adequate diets (2,000 to 2,500 cal, 80 to 100 g protein, 200 to 250 g carbohydrate, about 110 g fat daily) for 8 to 14 days, and the two regimens were compared. During the administration of the purified diet the patients showed no evidence of improvement either clinically, in liver function tests, or in liver fat content as estimated by examination of liver tissue obtained by needle biopsy. The provision of the adequate diet was consistently accompanied by improvement in hepatic function and decreases in liver fat content (Fig. 8).

These studies[55] made several contributions. Minimal conditions were attained under which patients with fatty liver associated with alcoholism would not demonstrate clinical improvement or improvement in liver histology. The therapeutic efficacy of an adequate diet was again obviously apparent. The fact that the patients studied did *not* improve while ingesting the purified diet, although they had coincidentally been hospitalized and withdrawn from alcohol, suggested that dietary factors were of primary importance to the improvement noted, and that rest in the hospital and alcohol withdrawal could be relegated to roles of minor significance.

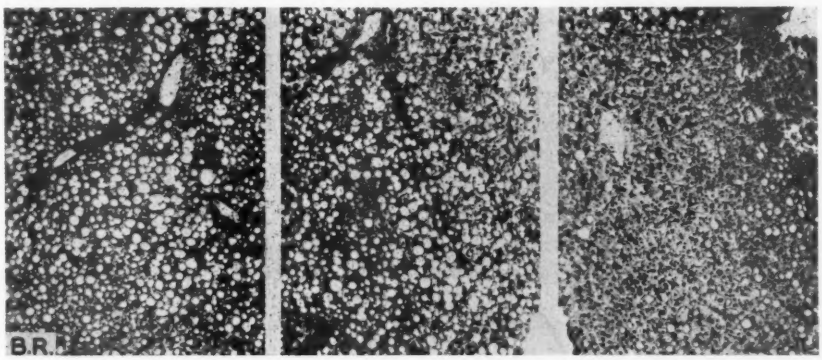


Fig. 8. The biopsies are in chronologic sequence from left to right. The first biopsy was performed on admission, the second after ten days of the purified diet, and the third after eight days of the adequate diet. Hematoxylin and eosin ×50. (From: Phillips, G. B., Gabuzda, G. J., and Davidson, C. S.: *J. Clin. Investigation* 31:351, 1952. Reproduced with the permission of the authors and publishers.)

The failure of these patients to improve while ingesting the purified diet offered the opportunity to evaluate critically agents of potential therapeutic value. The demonstration that patients with fatty livers would not improve clinically nor show a decreased liver fat content until choline was added to the "purified diet" regimen would provide strong evidence that choline was therapeutic, that it may be a factor contained in the nutritious diets that accounts for their beneficial effects, and that it may, by implication, be involved in the pathogenesis of this type of liver disease in man. Accordingly, five patients with fatty cirrhosis were studied initially while they subsisted on the purified diet to which choline was added, and then while

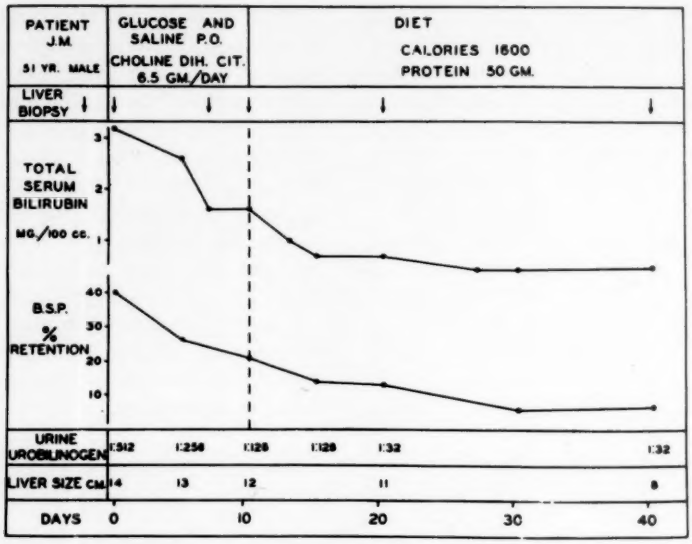


Fig. 9A. Clinical course of patient (J. M.). The biopsies are shown in Figure 9B.

they ingested diets containing 50 g of protein and 1,600 cal (isocaloric to the purified diet).[56] The results of these studies are illustrated by the following brief presentations.

One patient, J. M. (Fig. 9, A and B) was given the purified diet and 6.5 g of choline dihydrogen citrate orally daily for ten days. During this initial period of observation the serum bilirubin concentration, the bromsulphalein retention, the excretion of urobilinogen in the urine, and liver size decreased. Improvement by these criteria continued during the 10-day period on the well-balanced diet. Serial liver biopsies revealed initially a marked degree of fatty change, a "definite but not marked" decrease in liver fat after 10 days of the purified diet with choline, and a further decrease in liver fat content after 10 days of the

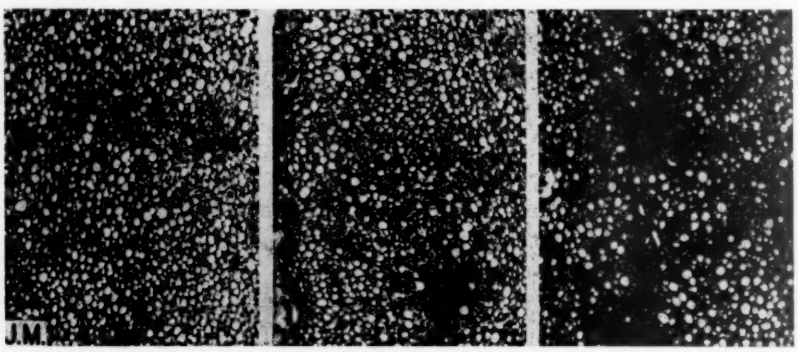


Fig. 9B. The biopsies (J. M.) are in sequence from left to right. The first biopsy was done on admission; the second, after 10 days of the purified diet and choline; and the third, after 10 days of the 1,600 calorie-50 g protein diet. (From: Phillips, G. B. and Davidson, C. S.: *Ann. New York Acad. Sc.* 57: 812, 1954. Reproduced with the permission of the authors and publishers.)

well-balanced diet (Fig. 9). This study might be interpreted as demonstrating a favorable effect of orally administered choline.

All the patients studied, however, did not demonstrate similar results. For example, another patient, C. W., similarly studied (Fig. 10) did not improve until a diet providing 50 g of protein daily was instituted. Of the five patients so studied, two demonstrated responses

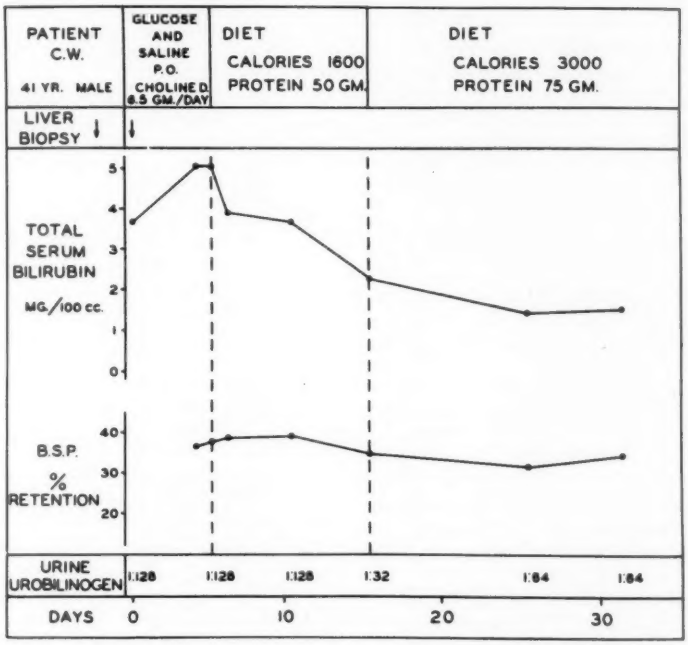


Fig. 10. Clinical course of a man with fatty liver given a purified diet supplemented with choline followed by an isocaloric well-balanced diet, and then a normal diet. (From: Phillips, G. B. and Davidson, C. S.: *Ann. New York Acad. Sc.* 57: 812, 1954. Reproduced with the permission of the authors and publishers.)

to choline which might be interpreted as favorable, and three had no convincing beneficial effect from the administration of this agent. Improvement did occur in these three patients following the administration of the 50 g protein, 1,600 cal diet. The reasons for the different responses noted in these patients are not known. These results do not necessarily mean that choline is not concerned in the pathogenesis of fatty liver in man; but neither do they conclusively incriminate it. They do provide additional evidence that a dietary factor(s) is responsible for improvement of fatty liver disease in man, and suggest that calorie intake *per se* is not critical. The favorable response of the patients reported by Phillips and Davidson[56] to the relatively meager rations employed in their investigations indicate that excessive intakes of food are not prerequisite for improvement (Fig. 1).

CONCLUSIONS

Nutritional factors may well be implicated in the pathogenesis and treatment of fatty cirrhosis of the liver associated with alcoholism. The clinical studies presented above that consistently demonstrate the therapeutic value of well-balanced diets further attest to this hypothesis. The problem of investigating the possible role of specific dietary factors, e.g., choline, methionine, etc., in the pathogenesis and therapy of fatty liver disease in man is a

difficult one. Although several encouraging experimental approaches have been employed, and have yielded information of value, to date they have failed to provide convincing evidence that lipotropic agents are involved in the pathogenesis or therapy of liver disease in man. Until evidence is presented to the contrary, patients with fatty liver receive optimal nutritional therapy if they are given adequate quantities of protein and other nutrients in well-balanced diets.

REFERENCES

1. Best, C. H., Huntsman, M. E., and Ridout, J. H.: The lipotropic effect of protein. *Nature* 135: 821, 1935.
2. Sellers, E. A., Lucas, C. C., and Best, C. H.: Lipotropic factors in experimental cirrhosis. *Brit. M. J.* 1: 1061, 1948.
3. Best, C. H., Lucas, C. C., and Ridout, J. H.: The lipotropic factors. *Ann. New York Acad. Sc.* 57: 646, 1954.
4. György, P. and Goldblatt, H.: Further observations on production and prevention of dietary hepatic injury in rat. *J. Exper. Med.* 89: 245, 1949.
5. Himsworth, H. P. *Lectures on the Liver and its Diseases.* Harvard Univ. Press, Cambridge, Mass., 1950.
6. György, P.: The nutritional aspects of liver injury. *M. Clin. North America* 33: 1657, 1949.
7. Rich, A. R. and Hamilton, J. D.: Experimental production of cirrhosis of liver by means of deficient diet. *Bull. Johns Hopkins Hosp.* 66: 185, 1940.
8. Patek, A. J., Jr. and Post, J.: Treatment of cirrhosis of the liver by a nutritious diet and supplements rich in Vitamin B complex. *J. Clin. Investigation* 20: 481, 1941.
9. Patek, A. J., Jr., Post, J., Ratnoff, O. D., Mankin, H., and Hillman, R. W.: Dietary treatment of cirrhosis of liver: Results in 124 patients observed during ten-year period. *J.A.M.A.* 138: 543, 1948.
10. Nutritional Factors and Liver Diseases. *Ann. New York Acad. Sc.* Vol. 57, 1954.
11. Ralli, E. P., Paley, K., and Rubin, S. H.: The liver lipids and their distribution in disease. An analysis of 60 human livers. *J. Clin. Investigation* 20: 413, 1941.
12. Zelman, S.: The liver in obesity. *A. M. A. Arch. Int. Med.* 90: 141, 1952.
13. Levin, L.: The possible involvement of the adrenal cortex and thyroid in mobilization of fat to the liver. *J. Clin. Endocrinol.* 9: 657, 1949.
14. Best, C. H.: *Diabetes and Insulin and the Lipotropic Factors.* Thomas, Springfield, Ill., 1948.
15. Iversen, K. and Ashoe-Hansen, G.: Studies on the fat-mobilizing factor of the anterior pituitary gland suppressive action of thyroxin. *Acta Endocrin.* 11: 111, 1952.
16. Levin, L. and Farber, R. K.: Relation of cortisone pretreatment to mobilization of lipids to liver by pituitary extracts. *Proc. Soc. Exper. Biol. & Med.* 74: 758, 1950.
17. Levin, L. and Farber, R. K.: Hormonal factors which regulate the mobilization of depot fat to the liver. *Recent Progress Hormone Res.* 7: 399, 1952.
18. Zimmerman, H. J., MacMurray, F. G., Rappaport, H., and Alpert, L. K.: Studies of the liver in diabetes mellitus. *J. Lab. & Clin. Med.* 36: 912, 1950.
19. Warren, S.: *The Pathology of Diabetes Mellitus.* Lea and Febiger, Philadelphia, 1938.
20. Reinberg, M. H. and Lipson, M.: The association of Laennec's cirrhosis with diabetes mellitus. *Ann. Int. Med.* 33: 1195, 1950.
21. Jaques, W. E.: The incidence of portal cirrhosis and fatty metamorphosis in patients dying with diabetes mellitus. *New England J. Med.* 249: 442, 1953.
22. Chaikoff, I. L.: The application of labeling agents to the study of phospholipid metaboiism. *Physiol. Rev.* 22: 291, 1942.
23. Artom, C.: Role of choline in the oxidation of fatty acids by the liver. *J. Biol. Chem.* 205: 101, 1953.
24. Thannhauser, S. J. and Reinstein, H.: Fatty changes in the liver from different causes. *Arch. Path.* 33: 646, 1942.
25. Leevy, C. M., Zinke, M. R., White, T. J., and Gnassi, A. M.: Clinical observations on the fatty liver. *A. M. A. Arch. Int. Med.* 92: 527, 1953.
26. Brock, J. W.: Survey of the world situation on kwashiorkor. *Ann. New York Acad. Sc.* 57: 696, 1954.
27. Gillman, J. and Gilbert, C.: Aspects of nutritional liver disease—human and experimental. *Ann. New York Acad. Sc.* 57: 737, 1954.
28. Hartroft, W. S.: The sequence of pathologic events in the development of experimental fatty liver and cirrhosis. *Ann. New York Acad. Sc.* 57: 633, 1954.
29. Hartroft, W. S.: The diagnostic significance of fatty cysts in cirrhosis. *A. M. A. Arch. Path.* 55: 63, 1954.
30. Popper, H., Szanto, P. B., and Elias, H.: Transition of fatty liver into cirrhosis. *Gastroenterology* 28: 183, 1955.
31. Chalmers, T. C., Murphy, T. L., and Taft, E. B.: The incidence, character and course of liver disease on chronic alcoholics as determined by needle biopsy. *J. Clin. Investigation* 27: 528, 1948.
32. Klatskin, G. and Yesner, R.: Factors in the

treatment of Laennec's cirrhosis. I. Clinical and histological changes observed during a control period of bedrest, alcohol withdrawal and a minimal basic diet. *J. Clin. Investigation* 28: 723, 1949.

33. Eckhardt, R. D., Zamcheck, N., Sidman, R. L., Gabuzda, G. J., and Davidson, C. S.: Effect of protein starvation and of protein feeding on the clinical course, liver function, and liver histochemistry of three patients with active fatty alcoholic cirrhosis. *J. Clin. Investigation* 29: 227, 1950.
34. Buck, R. E.: Observations on alcoholic fatty liver: The use of interval needle biopsy and liver function test. *J. Lab. & Clin. Med.* 33: 555, 1948.
35. Seife, M., Kessler, B. J., and Lisa, J. R.: Clinical, functional and needle biopsy study of the liver in alcoholism. *A. M. A. Arch. Int. Med.* 86: 658, 1950.
36. Post, J., Benton, J. G., Breakstone, R., and Hoffman, J.: The effects of diet and choline on fatty infiltration of the human liver. *Gastroenterology* 20: 403, 1952.
37. Cornatzer, W. E. and Cayer, D.: Effects of lipotropic factors on phospholipid turnover in plasma of patients with cirrhosis of liver as indicated by radioactive phosphorus. *J. Clin. Investigation* 29: 542, 1950.
38. Cayer, D. and Cornatzer, W. E.: Radioactive phosphorus as indicator of role of phospholipid formation in patients with liver disease. *Gastroenterology* 14: 1, 1950.
39. Cornatzer, W. E.: Phospholipid synthesis in liver metabolism. *Ann. New York Acad. Sc.* 57: 919, 1954.
40. Cornatzer, W. E. and Cayer, D.: Phospholipid synthesis in patients with cirrhosis and infectious hepatitis. *South. M. J.* 43: 212, 1950.
41. Cayer, D. and Cornatzer, W. E.: The effects of lipotropic factors on the phospholipid turnover in patients with infectious hepatitis. *Gastroenterology* 18: 79, 1951.
42. Williams, J. O., Cayer, D., and Cornatzer, W. E.: Lipotropic factors in treatment of cirrhosis: Evaluation by biopsy and by study of phospholipid turnover employing radioactive phosphorus. *South. M. J.* 44: 369, 1951.
43. Cayer, W. D. and Cornatzer, W. E.: The use of lipotropic factors in the treatment of liver disease. *Gastroenterology* 20: 385, 1952.
44. McMurray, C., Cayer, D., and Cornatzer, W. E.: Chronic adhesive pericarditis due to the rheumatic state, associated with liver damage, serous effusions and pigmentation. *Gastroenterology* 17: 294, 1951.
45. De la Huerga, J. and Popper, H.: Factors influencing choline absorption in the intestinal tract. *J. Clin. Investigation* 31: 598, 1952.
46. De la Huerga, J. and Popper, H.: Urinary excretion of choline metabolites following choline administration in normals and patients with hepatobiliary diseases. *J. Clin. Investigation* 30: 463, 1951.
47. De la Huerga, J., Popper, H., and Steigmann, F.: Urinary excretion of choline and trimethylamines after intravenous administration of choline in liver diseases. *J. Lab. & Clin. Med.* 38: 904, 1951.
48. Kinsell, L. W., Harper, H. A., Barton, H. C., Hutchin, M. E., and Hess, J. R.: Studies in methionine and sulfur metabolism: I. The fate of intravenously administered methionine in normal individuals and in patients with liver damage. *J. Clin. Investigation* 27: 677, 1948.
49. Kirsner, J., Sheffner, A. L., Palmer, W. L., and Bergeim, O.: Amino acids in plasma and urine of patients with hepatitis before and after a single infusion of protein hydrolysate. *J. Lab. & Clin. Med.* 36: 735, 1950.
50. Eckhardt, R. D., Cooper, A. M., Faloon, W. W., and Davidson, C. S.: The urinary excretion of amino acids in man. *Tr. New York Acad. Sc.* 10: 284, 1948.
51. Dunn, M. S., Akawaie, S., Yen, H. L., and Martin, H. E.: Urinary excretion of amino acids in liver disease. *J. Clin. Investigation* 29: 302, 1950.
52. Gabuzda, G. J., Eckhardt, R. D., and Davidson, C. S.: Urinary excretion of amino acids in patients with cirrhosis of the liver and in normal adults. *J. Clin. Investigation* 31: 1015, 1952.
53. Chalmers, T. C. and Davidson, C. S.: Survey of recent therapeutic measures in cirrhosis of the liver. *New England J. Med.* 240: 449, 1949.
54. Eckhardt, R. D., Faloon, W. W., and Davidson, C. S.: Improvement of active liver cirrhosis in patients maintained with amino acids intravenously as a source of protein and lipotropic substances. *J. Clin. Investigation* 28: 603, 1949.
55. Phillips, G. B., Gabuzda, G. J., and Davidson, C. S.: Comparative effects of a purified and an adequate diet on the course of fatty cirrhosis in the alcoholic. *J. Clin. Investigation* 31: 351, 1952.
56. Phillips, G. B. and Davidson, C. S.: Nutritional aspects of cirrhosis in alcoholism—effect of a purified diet supplemented with choline. *Ann. New York Acad. Sc.* 57: 812, 1954.

DISCUSSION

Dr. Labecki: I am glad that Dr. Gabuzda stressed the multiplicity of factors which may be responsible for the fatty infiltration of the liver in the alcoholic, and the fact that the deficiency of possibly more than one substance or group of substances may play a role in the etiology of the disease.

I profess to be a cardiologist. However, Dr. Busby and I got interested in the problem of fatty liver in

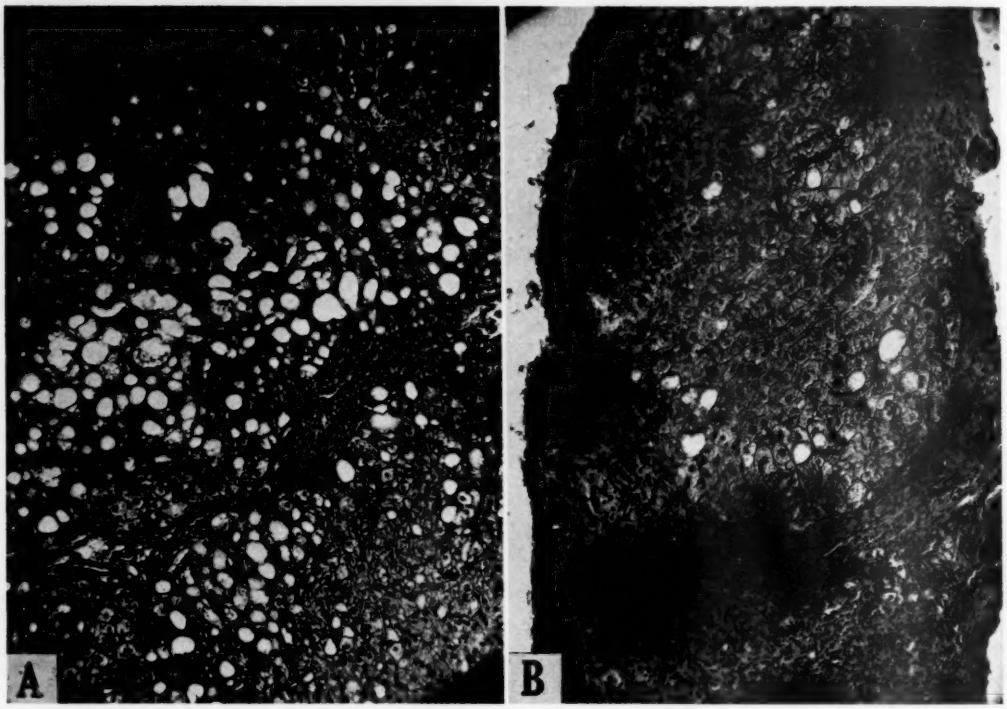


Fig. 1A. N. I., white male, age 44. Drinking 25 years, often one-fifth per day. Liver hard, nodular on palpitation; enlarged 7 cm BSP 4% (45 min); thymol turbidity 1.7 Maclagan units; cephalin flocculation 1+. (Abundant glycogen.) (150×) Fig. 1B. N. I., four weeks on Regimen A including placebo. BSP 4% (45 min). (Decreased amount of glycogen.) (160×)

alcoholics for two reasons (1) a long-term interest in fat metabolism; (2) the fact that the State of Mississippi is a "dry" state and hence has excellent material for the study of alcoholism. I do not say that entirely in the spirit of levity, because I feel that a dry state offers an inducement to drink by the bottle rather than at the bar.

The thing which impressed me was that our alcoholic patients differed considerably from the type of alcoholics I encountered in Chicago and Los Angeles: a typical alcoholic in our state is not a derelict. He usually comes to the Alcoholic Ward of our State Hospital well nourished, or at least it appears so.

I would like to present to you some of the findings of a study of the role of lipotropic factors in the treatment of alcoholic fatty liver which we have exhibited before the American Medical Association (Atlantic City, June 6–10, 1955).

This 44-year-old male had been drinking whiskey for 25 years, often a fifth daily (I must admit that we have had alcoholics in better standing in our series, that is, the daily consumption of alcohol was even greater than in this particular case). These patients came in voluntarily, and as long as they were willing to cooperate they received the benefit of any "treatment" we chose to administer; they could sign out at any time. We started with 54 patients, but only 30 elected to carry through the experiments for a sufficiently long period of time. This particular gentleman whose liver biopsy is shown in Figure 1A, was first put on so-called Regimen A which consisted of a conventional high-protein diet supplemented by cooked egg white (12 per day), skim milk, and cottage cheese *ad lib.*, therapeutic multiple vitamin preparation and placebo (lactose capsules). The protein content of the daily diet in some cases reached as high as 150 g of protein daily and the consumption of the diet was supervised and insisted upon by ward attendants. Figure 1B shows definite improvement in the degree of fatty infiltration but still one can easily detect the presence of vacuoles. The duration of Regimen A in this case was four weeks.

The liver biopsy specimen (Fig. 1C) shows the results of five weeks of so-called Regimen B which consisted of a moderate calorie diet (up to 2,300 calories per day) containing only a modest amount of protein, not over

40 g/day, but supplemented by relatively high intake of lipotropic factors administered in many cases parenterally (in addition to oral medication consisting of 9 capsules daily of Methiscol® which provided 1.0 g of DL-methionine, 2.5 g of choline dihydrogen citrate [0.87 g. of choline], 0.75 g of inositol, 18 μg of vitamin B_{12} and 0.78 g of desiccated liver concentrate). The disappearance of fatty infiltration from liver parenchyma is obvious.

The next case is that of a chap 41 years of age. This gentleman had been drinking whiskey for 20 years and according to the members of his immediate family,

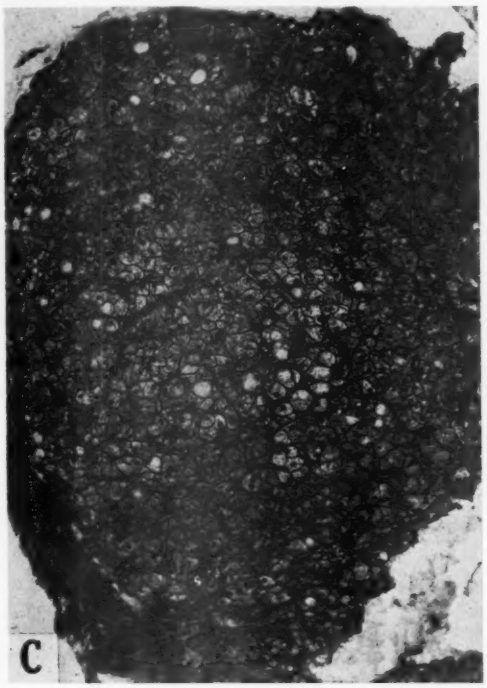


Fig. 1C. N. I., five weeks on Regimen B (Methischol® oral and parenteral). BSP 0% (30 min). (Abundant glycogen.) (175×)

on a good week end he could consume up to a gallon of hard liquor in a 24-hour period. His case was obviously exceptional from the amount of alcohol consumed, which fact amply reflects itself in the appearance of his liver parenchyma (Fig. 2A). In his case, as in several other cases, we did not dare to administer high-protein diet because of the possibility of precipitating hepatic coma, but instead administered lipotropic therapy for eight weeks. After two months his liver shrank and became so hard that it was impossible to obtain biopsy with the Vim-Silverman needle and the specimen had to be obtained through peritoneoscope.

Fig. 2B shows the appearance of the liver at that time. There is complete disappearance of fatty infiltration.

An argument may be advanced that the complete relief of fatty infiltration following intensive lipotropic therapy is the carry-over effect from the high-protein, high-vitamin regimen. However, we have treated patients with a high-protein, high-vitamin therapy for periods up to 22 months without resolution of the fatty infiltration upon institution of lipotropic therapy, the fat disappeared from the liver in less than two months.

When we started our study in 1952, the high-protein diet still enjoyed the popularity and was indeed the treatment of choice. Doctors Davidson, Gabuzda, and collaborators (Phillips, Schwartz, Gabuzda, and Davidson, *New Eng. J. Med.* 247: 239, 1952) brought to our attention the inherent danger of high-protein diet.

We have treated patients with advanced Laennec's cirrhosis with the high-protein diet (Regimen A) and have precipitated fatal hepatic coma resistant to all therapy. On the basis of these data we believe that the dietary treatment of choice for alcoholic fatty liver is a moderate protein diet supplemented with lipotropic factors.

Dr. L. Freedman (U. S. Vitamin Corporation, New York): This discussion of the problem the physician has to contend with in the treatment of liver disease recalls to mind a visit I made to the government hospital in Haifa (Israel) in 1950. At that time there was an extremely high incidence of liver disease associated with starvation. Nutritional jaundice was prevalent in these patients. They had large livers, and they were extremely sick. The dietary problem was acute. There was no possible way of treating these patients with high-protein diet; there was just not enough food to go around. The hospital beds were filled with these patients coming in, most of them immigrants from Saudi Arabia, Morocco, and other countries. The average time of the patient in bed was six weeks and sometimes a great deal longer. They did not do liver biopsies since they had no means of doing that. I brought in the only Vim-Silverman needles that they had in the entire city of Haifa.

I had sent them several thousand capsules of a preparation comprising a mixture of lipotropic products. They used these to treat the patients, and "miraculously" they were able to get the patients out of bed in about three weeks, still without any high-protein or high-caloric intake of food. As a matter of fact, these patients were too sick to eat. While this is not a scientific account of the pathogenesis of liver disease and the prognosis with a given treatment, it is a factual incident in the treatment of human patients who were extremely sick and among whom there was a very high incidence for morbidity and relatively high mortality prior to lipotropic treatment.

Dr. Wilgram: Dr. Gabuzda, I have two technical questions. One, did you say cirrhosis is more prevalent in alcoholic patients who are well nourished than in those who are badly nourished?

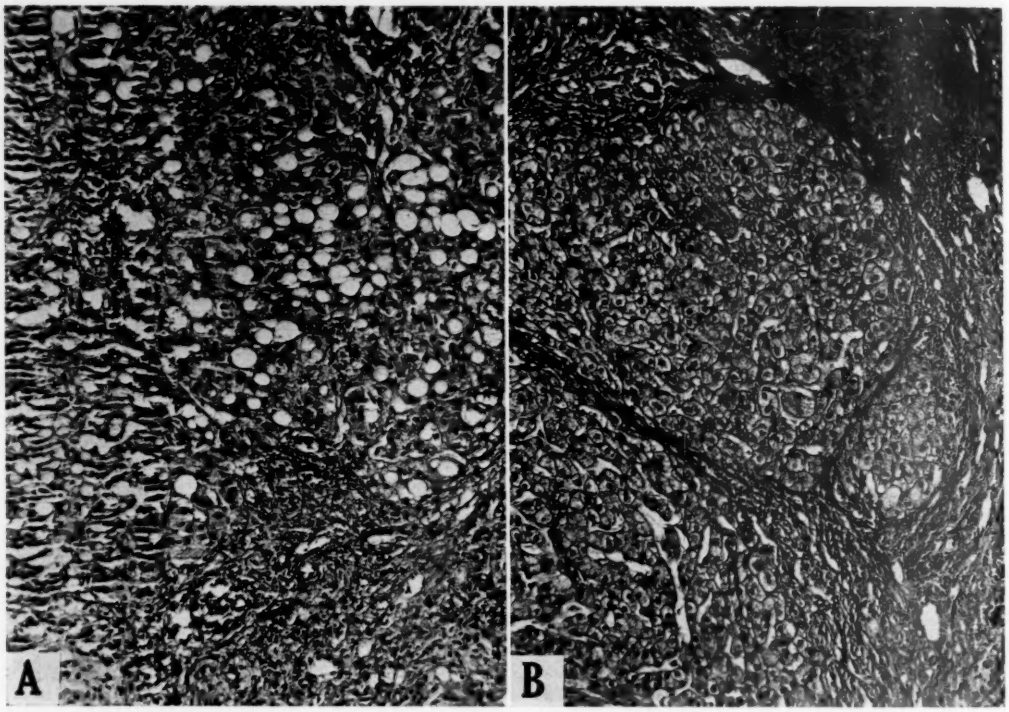


Fig. 2A. S. H., white male, age 41; drinking for 20 years (consumed up to a gallon per 24 hours for past 8 years). Liver hard and smooth on palpation but nodular on peritoneoscopic examination. Liver enlarged about 9 cm. BSP 22% (45 min); thymol turbidity 1.9 Maclagan units; cephalin flocculation 4+. (175×) Fig. 2B. S. H., eight weeks of intensive lipotropic therapy (Methischol®). BSP 14% (45 min). Improvement of liver on palpation. (175×)

The other, you mentioned glycogen synthesis seems to be at the normal rate in patients on protein-poor diets. I was wondering how this agrees with the older statements that glycogen synthesis is one of the first liver functions to be affected in patients on protein-poor diets and with certain other diseases.

Dr. Sheffner: Would Dr. Gabuzda comment on the difference in response between patients receiving the glucose diet and those receiving glucose plus fat, especially since the clinical improvement on the latter diet occurred in the absence of morphologic improvement.

Dr. Gabuzda (closing remarks): I suspect Dr. Labecki and I are talking about two more or less different types of patients. His patients are patients who have been drinking steadily over quite long periods of time and have, coexistent with increased fat content, hepatic fibrosis. The fat is admittedly more difficult to remove from the livers of these individuals than in the case of the spree drinker type I had mentioned whose lesions are usually reversible. We were interested in dealing with the reversible lesions in attempting to determine whether or not a therapeutic effect could be accomplished in a period of time reasonable enough to assure that a possible deficiency of lipotropic agents might be corrected.

I wonder if Dr. Labecki in his studies followed any patients on a "low-protein regimen" without supplementary lipotropic substances as an additional control group.

Many of our patients are well nourished. Some of them are obese. They may appear healthy except for the clinical and laboratory evidence of liver disease.

The reports of the treatment of liver disease in Haifa are interesting. The only clinical situation in which there is any rationale for the use of lipotropics is in the treatment of fatty liver disease. It may well be that in these situations in which patients are not able to eat even meager rations, lipotropics may be advantageous. On the other hand, we have demonstrated that relatively meager ratios which can usually be successfully applied in this country seem to accomplish as much as is needed.

In reference to the question about glycogen, liver

glycogen stores in these patients were somewhat decreased when they came to the hospital, and glycogen was repleted in the liver even during the periods when dietary protein was insufficient. The patients apparently were able to replenish liver glycogen stores in face of protein starvation.

There is a difference in the response of the patients given glucose plus fat as sources of calories as compared to those given glucose, water, and salt alone. The reasons for this are not entirely apparent. We could not be sure, for instance, that the less purified diet did not provide some agents that were of possible therapeutic value, possibly even traces of agents with lipotropic activity, or that the patients did not have the opportunity to partake of some food before the studies were initiated. For such reasons we did the subsequent experiments to limit the situation even further. We thus had an experimental setup under which improvement did not occur; and we were in a better position to test the efficacy of specific agents.

Dr. Jack D. Myers (University of Pittsburgh, Pittsburgh, Pa.): Dr. Labecki, would you care to answer Dr. Gabuzda's question regarding the control of the regimen? That is, did you treat any of your patients with the low-protein regimen omitting the lipotropic agent?

Dr. Labecki: Yes. We used placebos during the introductory phase of the study. We administered high-protein diets to one group and we also carried four patients on a low-protein diet with placebos. Admittedly, hospitalization in itself has a salutary effect. They improved from the standpoint of the degree of fatty infiltration. They apparently never improved, at least our patients did not, to the point which those did who received maximum lipotropic therapy.

Dr. Olson: Did you conclude that the improvement with 50 g of protein plus lipotropic therapy was superior to that of 50 g of protein alone?

Dr. Labecki: Yes, superior. Besides, after our experience with the patient who went into hepatic coma following the administration of high protein feedings, possibly due to an inability to convert ammonia to urea (Davidson, *Am. J. Med.* 16: 863, 1954), we shy away from high-protein diets.

Fatty Liver in Children—Kwashiorkor

SILVESTRE FRENK, M.D., FEDERICO GÓMEZ, M.D., RAFAEL RAMOS-GALVÁN, M.D., AND JOAQUÍN CRAVIOTO, M.D.*

POINTS of view have changed since Waterlow in his famous monograph[1] considered the fatty liver of children with nutritional edema and muscular wasting as a fundamental lesion and the one mainly responsible for a fatal outcome. The same author,[2] through more adequate measurements of the fat content of the liver in a much greater number of malnourished infants, has demonstrated that this original conclusion cannot be maintained. It seems to be clear now that liver steatosis is not responsible for the signs and symptoms of the malnutritional syndrome,[3] and that its significance probably does not go beyond that of being a phenomenon incidental to severe malnutrition.

Fatty liver in children represents perhaps the most widespread natural manifestation of the interaction of the various factors so far discussed at this meeting. However, since the evidence incriminating dietary factors in the pathogenesis of liver disease is necessarily more specific in laboratory animals than in man,[4] it may seem somewhat risky to attempt an integration of those factors into a particular scheme of the mechanism of fatty liver in malnourished infants, according to our present knowledge.

Regardless of the cause of the nutritional disturbance, liver steatosis may occur equally in primary dietary malnutrition and in conditions secondary to any acute or chronic wasting disease. Only the picture as it appears in most cases of chronic severe infantile malnutrition will be described here.

According to classic observations made in tropical areas such as the West Indies[1] or Uganda,[5] the liver is enlarged in a considerable proportion of all children affected with chronic severe malnutrition. But while the fundamental features of the syndrome are the same all over the world, clinical hepatomegaly is an exception. On the high Mexican plateau, for instance, only about 10 per cent of the seriously malnourished children have a palpable liver, although, hepatomegaly appears some weeks later as one of the main signs of the Nutritional Recovery Syndrome.[6] Autopsies done in Mexican patients who die soon after their admission, have shown that in one-third of them the liver weighs more than normal, while in the others it participates in the over-all decrease in organ weight typical of malnutrition. About 70 per cent of these livers show fatty changes,[7] together with a definite atrophy if allowance is made for fat content.[2]

Fatty liver accompanying chronic severe infantile malnutrition has been described many times, and only superficial mention will be made here of its main characteristics.

PATHOLOGIC FINDINGS IN THE LIVER

The organ is described as being yellow and friable; the cut surface greasy, bulging, of an orange-yellow color with red mottling, due to premortem dilatation of the central veins.[3]

Microscopically, wide variations in the fat distribution may be found. Steatosis generally appears first at the periphery of the hepatic lobule, and from there it progresses toward the central vein until the whole organ is invaded. The initially small fat droplets coalesce later until the whole cell is distended by a large fat globule and the cytoplasmic components and the nucleus are pushed to the periphery (Fig. 1). Sometimes, so-called lipodiastemata, that is, fusion of several fatty cells, may be seen. This characteristic pattern[5] may become modified if malnutrition is complicated by some serious infective process in which fat deposition may be irregular, in distribution and in amount.[8]

* Group for Research on Infantile Malnutrition, Hospital Infantil, México, D.F.

Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc.

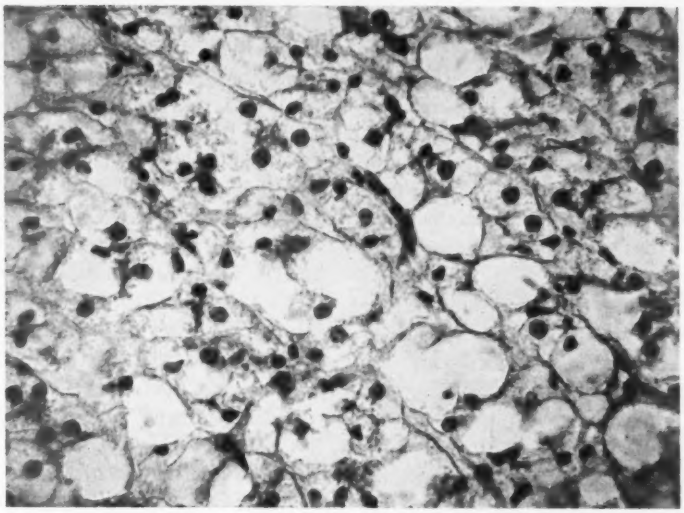


Fig. 1. Fatty liver in chronic severe infantile malnutrition.

When fat disappears from such a liver, it does so in the reverse order of its appearance, first from the centrolobular zone and last of all from the periphery. At its place, a watery glycogenic vacuolation becomes visible, producing a foamy or empty cell appearance.[9] This particular picture is typical of the liver during nutritional recovery (Fig. 2).

Other histologic findings include:

Liver sinusoids are compressed and the organ contains very little blood in about 20 per cent of the cases with severe fatty infiltration. As steatosis advances, the portal triads are invaded by round cells, mostly lymphocytes and plasmocytes. Fat is then disappearing from the liver; it is the sinusoids which are literally flooded with these cells.[10] An attempt has been made to correlate these cellular changes with the presence on one hand of lipolytic enzymes in the lymphocytes, and on the other

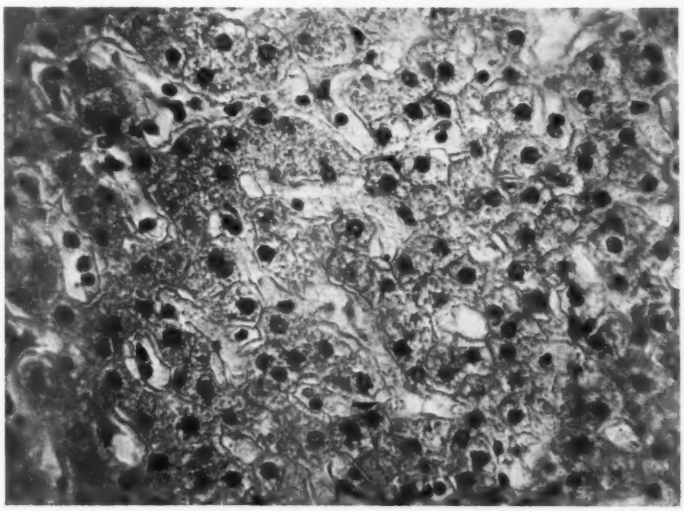


Fig. 2. The liver during recovery from chronic severe infantile malnutrition.

with the abnormal serum gamma-globulin levels of malnutrition.[5]

In older children, over four years of age, fatty metamorphosis is usually less conspicuous; about half of them show an increase of connective tissue, manifested as fine bands wound around the hepatic lobules. A star-like pattern of fibrosis may develop, and most observers believe that it is independent of steatosis.

Pigmentation of the liver[3] is found fairly frequently, more in younger children, and in our series is not specific for malnutrition. In most cases the pigment is iron-containing, but chromolipoid is almost always present simultaneously.[7]

In recent years, an increasing importance has been attached to pancreatic lesions, occurring in association with nutritional liver disease.

Disappearence of zymogen granules has been considered as a phenomenon specific for "kwashiorkor,"[10] but is also found in children of the "marasmic" type of malnutrition,[11] who, as shown later, may have no fatty liver at all. On the other hand, a rather high degree of association between pancreatic changes and intensity of steatosis is found in non-primarily-nutritional fatty liver.[12] In later stages pancreatic fibrosis may be present.

Tissue Analyses: It seems obvious that chemical analysis of liver tissue should give a more objective index of its composition than histologic methods, provided one has an adequate reference. The importance of the latter becomes apparent when dealing with very fatty livers in which the usual expression of fat as per cent is likely to underestimate the severity of steatosis.[12,13] Therefore, in biopsy specimens where the weight of the organ cannot be known, it is convenient to relate liver fat to dry non-fat solids.[13]

Except when fatty infiltration is maximal, i.e., on the order of 50 per cent of the net weight of the organ, there is no correlation between the fat content of the liver and the clinical condition or the prognosis of the patient.[14] A malnourished child may be on his way to recovery long before he starts to lose his liver fat, and another may be seriously ill without showing any abnormal amounts of hepatic lipids.

Definite correlations have been established between the degree of steatosis and certain features characterizing the extreme clinical pictures to be found in chronic infantile malnutrition: the "Mehlnährschaden" or "kwashiorkor" baby on one hand, the "atrophic" or "marasmic" on the other and the "marasmic kwashiorkor" in between. In our series, the first group shows a mean age of 38 ± 18 months when admitted to the hospital, with a history of over-all nutritional deficiency since the age of weaning and a relative adequate caloric intake. In most cases there has been a fairly recent acute infectious episode, and/or repeated bouts of severe gastrointestinal disturbances. Generally, there is pitting edema and characteristic skin lesions are frequently found. Although these children are 40 per cent or more underweight and show gross muscular wasting, nevertheless, considerable amounts of subcutaneous fat can be detected on the cheeks, the abdomen, and the thighs. These children show the greatest incidence and quantity of fat in the liver.

Survivors of this stage of infantile malnutrition may show a gradual disappearance of both body and liver fat deposits,[10] drifting through the stage of "marasmic kwashiorkor" into typical "marasmus." Younger children may show only the later aspect, as suggested by a mean age of 23 ± 15 months for the "atrophic" group. At any rate, in spite of the striking difference in appearance, over-all mortality has been found the same in all clinical types.[15]

Evidence points to a rough correlation between the amount of fat in the liver and the fat content of the body.[16] A significant association has been found between presence of pitting edema and of a severely fatty liver,[13] although the latter may be found in the absence of the former. Recent study of the composition of skin plus subcutaneous tissue[17] has shown that regardless of the presence or absence of pitting edema there is the same excess of water in most of the cases. The difference lies in the amount of fat per unit of dry, fat-free solids which suggests that in infantile malnutrition recognition of clinical edema depends upon the presence of a good-sized subcutaneous fat pad beneath an overhydrated skin. It is this which seems to

correlate with the amount of liver fat rather than clinical edema.

Regardless of the amount of fat in the liver, what seems to be a common denominator in all cases of chronic severe infantile malnutrition is the very small amount of cytoplasm. A special significance may thus be attached to the latter.

It is well known that when rats are starved or placed on a protein-free diet, they lose up to 40 per cent of their total liver protein and part of their ribonucleic acid and phospholipids in a matter of a few days.[18] If malnutrition is prolonged, the brunt of protein loss falls, not on the liver or other internal organs with a high protein turnover, but on the muscles. It is to be expected, therefore, that "protein depletion" of the body might be better shown by analyses of muscle than of the liver.[14]

When, as in studies on human beings *in vivo*, it is not possible to measure the total weight of an organ, the loss of cytoplasmic protein may be demonstrated by relating protein content to nuclear desoxyribosenucleic acid (DNA), assuming that the amount of the latter present per cell and per liver remains constant.[14] The results obtained with this approach[19] are shown in Tables I and II. It must be taken into account that during recovery from malnutrition, there is a tendency to nuclear poliploidy, and that therefore the premise of a constant amount of DNA may be subjected to doubt.[16]

TABLE I

Liver Fat and Liver Protein at Autopsy (Waterlow, 1956)

	Kwashiorkor	Marasmus and mixed cases	Controls
Mean body weight (% of ideal wt)	58	51	79
Mean liver fat (% fresh wt)	35	15	15
Mean liver protein (% of expected)	67	56	80

It can be seen that by comparison with a normal or ideal standard,[20] the total amount of liver protein is reduced by nearly 40 per cent, both in "kwashiorkor" and "marasmic" cases.[13,14] In addition, in kwashiorkor, classically associated with a diet predominantly lacking in proteins, the protein content of the liver is higher the more fatty the organ is. In Table II it may be seen that during recovery, protein N in terms of DNA phosphorus increases markedly. If, as pointed out heretofore, DNA may rise by itself during recovery, this increase in total liver protein may be much greater than indicated by the change in the ratio N/DNA.

TABLE II

Fat, Protein, and Nucleic Acid Content of the Liver in 12 Cases of Kwashiorkor that Recovered (Waterlow, 1956)

	Initial	Final
Fat, % of wet wt	34	3
Protein N, μg/mg DFF wt	80	83
DNAP, μg/mg DFF wt	1.73	1.12
Protein N/DNAP	49	83

Additional data on the livers of these malnourished children show that aside from fat, there is also a considerable increase in water content and a high concentration of glycogen. Incomplete histochemical studies show that during recovery, glycogen and glycoproteins tend to increase further.

LIVER FUNCTION

It may be a difficult task to correlate results obtained on a few liver functions and knowledge of the patterns of some metabolic indices, with the known features of tissue composition. Most authors seem to agree that serum bilirubin concentrations are found within normal limits in chronic severe malnutrition.[21] No correlation has been found between a whole battery of turbidity and flocculation tests and the clinical condition of the children, as well as the amount of liver fat.[22] Abnormal cephalin-cholesterol tests are mostly related with the presence of some complicating infectious process, especially enteritis or otitis.[21] Thymol turbidity and flocculation tests become positive during recovery.[6]

The bromsulphalein test done with a dose of 5 mg/kg body weight, measured at a 45-min. interval, has shown an abnormal retention of 15 ± 9 per cent,[23] which cannot be demonstrated

on a lower dose. A definite prognostic significance has been attached to the persistence of this abnormal dye retention, since cases in which it does not become normal in a matter of a few days, almost invariably die. The abnormal abstraction of bromsulphalein has been related to compression of the liver sinusoids by the fat-laden cells,[1,24] in spite of the lack of correlation between structural changes in the liver and functional efficiency.[5]

A constant and characteristic feature of infantile malnutrition is an abnormal serum protein pattern. Data obtained on clinically edematous and non-edematous children are shown in Table III. Comparable results have been obtained employing chemical, immunologic and electrophoretic methods.[25] It may be definitely stated that hypoalbuminemia is one of the most constant biochemical features of chromic severe malnutrition whether or not associated with a fatty liver. The significant differences found between total protein, albumin and globulin in both clinical groups may be related to different patterns of distribution within the organism. With the feeding of an adequate diet, serum proteins return to normal quickly without affecting the rate of recovery.[26]

TABLE III

Serum Proteins in 395 Cases of Chronic Infantile Malnutrition

	Children with clinical edema			Children without clinical edema		
	M*	S.D.†	S.E.‡	M	S.D.	S.E.
Total proteins	4.36	1.03	0.05	5.63	0.90	0.10
Albumin	1.64	0.70	0.04	2.59	0.70	0.08
Globulins	2.71	0.70	0.04	3.02	0.99	0.11
Alpha	0.71	0.38	0.02	0.83	0.31	0.04
Beta	0.80	0.50	0.03	0.86	0.42	0.05
Gamma	1.25	0.47	0.03	1.32	0.45	0.05

* M = mean; † S.D. = standard deviation; ‡ S.E. = standard error.

This, and the knowledge that the diet eaten by these patients meets about 50 per cent of the theoretic requirements for the age, both in amount and in biologic value, suggest that the main reason for the abnormalities lies in deficient protein synthesis in the liver, due to an insufficient provision for building amino acids. Moreover, it has been proved that absorption and retention of nitrogen are directly correlated with the nitrogen intake and with the biologic value of the ingested protein.[27]

Studies on the rate of synthesis and catabolism and of distribution of radioalbumin in the organism of malnourished children,[28] have shown that the total exchangeable albumin pool and circulating albumin are decreased in all cases, regardless of the presence or absence of edema. The fractional rate of catabolism or turnover rate, that is, that fraction of the total exchangeable pool that is catabolized per unit of time, has been found within the limits observed in normal children. The turnover half-time is apparently normal. Since there is no increase in catabolism nor abnormality in distribution, it would appear that in the severely malnourished child, hypoalbuminemia is due to a reduced hepatic synthesis, secondary to insufficient amino acid provision, as pointed out before.

A seemingly good index for liver function, apparently going parallel with albumin synthesis, is the production of cholinesterase in the liver. By means of the use of selective inhibitors of the enzyme (dialkylfluorophosphates) and infusions of albumin,[29] a close parallelism between the albumin and cholinesterase synthesis in the liver has been suspected. Studies on malnourished subjects have shown low values of acetylcholinesterase in erythrocytes and pseudocholinesterase in plasma,[26,30,31] with a fairly rapid increase upon recovery.

Since the cholinesterase molecule is a protein, its activity should reflect changes in one fraction of the serum proteins. However, there is no true correlation between the activity of this enzyme and the concentration of total serum proteins, nor is cholinesterase influenced by changes in body posture as are the proteins.[30] On the other hand, there is no correlation of this enzyme with the degree of fatty infiltration of the liver.[14]

These findings are in contrast to data obtained on rats fed hypolipotropic diets,[32] which show a rise of both plasma and liver cholinesterase and a return to normal when the dietary defect is corrected. This has suggested

a linkage between cholinesterase levels and certain phases of fat metabolism. Thus, a positive correlation between skinfold thickness and plasma cholinesterase has been found in adults.[33]

In human beings, the liver cholinesterase participates in similar fluctuations. Significant differences between values found on admission and five weeks later have been observed, the enzyme activity per cell increasing more than threefold upon recovery from malnutrition.[34]

Besides this nonspecific cholinesterase, only succinoxidase has been found reduced in livers of malnourished infants.[35] Tissue levels of other enzymes so far studied seem to be within normal limits, although "alkaline" phosphatase has been reported to be increased in the acute stage of the syndrome.[36]

The levels of this enzyme in the plasma follow a somewhat unusual pattern. Initial values are described as being below average; they continue dropping for about one week and subsequently show a steady rise to the upper levels of normal.[25,37] Since the blood level of "alkaline" phosphatase reflects, not only liver function but osteoblastic and general growth activities as well, and besides, is known to be depressed by an insufficient dietary intake, this curve is probably the resulting one of the integration of several factors. Moreover, it may also indicate the peculiar distribution and evolution of the magnesium-activated and the cyanide-resistant moieties of phosphatase, as described in malnourished babies.[38]

It has been pointed out that in such a disease as malnutrition one perhaps should not think in terms of amounts but of organization of an enzyme. It seems unlikely that measurement of the activity of a particular enzyme or enzyme system could be used as an index of chronic severe malnutrition, since the pattern of enzymatic activity in different organs is probably constantly changing as deficiency progresses.[35]

While so much is known about the role of amino acid deficiency and imbalance in the production of fatty livers in experimental animals, knowledge of amino acid metabolism in severely malnourished children is still scarce and fragmentary. Recovery may be initiated by feeding a mixture of synthetic amino acids.[39] Studies on urinary amino acid partition have shown a low threonine output, an absolute and relatively higher excretion of isoleucine than of leucine, and of phenylalanine greater than tyrosine.[40] Aminoaciduria has been observed during recovery. Recent data obtained in our laboratory in collaboration with R. Westall and L. E. Holt, Jr. show generalized hypoaminoacidemia, with a still greater decrease in tyrosine, phenylalanine, and alanine, while methionine and threonine are reduced in about the same proportion as total aminoacidemia. The exact significance of these findings is as yet unknown, particularly as regards the pathogenesis of liver steatosis. An impairment of phenylalanine conversion to tyrosine has been postulated, and the changes in hair color described in severe chronic infantile malnutrition are compatible with this hypothesis.[41]

Certain peculiarities of carbohydrate metabolism in these babies have been considered as manifestations of a failure in liver function. Malnourished children may die suddenly. Hypoglycemia is sometimes found, especially in association with infection, although the symptoms may not always point to that diagnosis.[42] Studies on carbohydrate-load tests have shown a consistent decrease in tolerance, both to dextrose and to galactose.[22,42,43,44] This pattern, comparable to Hofmeister's hunger diabetes, does not seem to follow a particular correlation with the clinical aspect of the child nor the histologic appearance of the liver. A failure of the liver cell to store glycogen when presented with abundant glucose administered intravenously has been observed,[43] even in the presence of satisfactory amounts of glycogen as judged by chemical or histologic analyses. Some children have been reported to show an inadequate rise of blood sugar after epinephrine injection; this feature also becomes more apparent during an infectious process.[42] Serum inorganic phosphorus and potassium determined during intravenous glucose tolerance tests show practically no fall along the whole curve.[45]

Considerable interest has been devoted to lipid metabolism in these children. For instance, preliminary observations on liver ho-

mogenates suggest that the rate of P^{32} uptake into phospholipids is strikingly decreased in malnourished livers.[46]

There is a uniform agreement among investigators of the subject that serum cholesterol is decreased in malnourished infants.[25,47,48] As in most other parameters studied, a quick rise characterizes initiation of recovery. A very high ratio of free-to-total cholesterol has been found, averaging 0.76 while in normals it is about 0.25. The latter value is rapidly reached when recovery starts. This ratio may represent an indirect way of estimating liver cholesterol esterase activity.

The concentration of total lipids in serum is also low in most cases. The subsequent rather quick attainment of normal values has been hypothetically related to removal of fat from its deposits, including a fatty liver, and was postulated as an explanation for the changes in the thymol tests found at recovery.[24] Exceptional cases, which also show an initially normal serum cholesterol, have high total lipids on admission, which later decrease. In all cases, there is a markedly decreased alpha-lipoprotein fraction which rapidly returns to normal.[48] Data for two representative cases are presented in Table IV.

TABLE IV

Blood Serum Lipids in Two Cases of Chronic Severe Malnutrition, as Found at Four-Day Intervals

No.	Total lipids *mg/100 ml*	Cholesterol *mg/100 ml*	α-Lipoproteins		β-Lipoproteins	
			% area	*mg/100 ml*	*% area*	*mg/100 ml*
1	272.8	111.3	6.25	17.0	93.75	255.8
2	300.2	113.2	6.75	20.2	93.25	280.0
3	328.6	107.7	10.35	34.0	89.35	294.6
4	357.5	126.4	16.85	60.2	83.15	297.3
5	383.7	151.1	11.03	42.3	83.97	341.4
6	462.1	177.7	15.77	72.8	84.23	389.2
1	574.0	142.5	5.72	32.8	94.28	541.1
2	514.0	148.0	7.10	36.5	92.90	477.4
3	391.9	159.1	7.56	29.6	92.44	362.2
4	388.2	169.1	10.50	40.7	89.50	347.4
5	395.6	159.1	13.47	53.3	86.53	342.3

It may be recalled that normal adults living in technically underdeveloped countries show a comparable pattern in their serum lipid fractions.[49] Concentrations of cholesterol and $-S_{1.21}$ 12-20 lipoproteins (which partially correspond to α-lipoproteins as determined in our laboratory[50]) are considerably lower in inhabitants of these countries than in North Americans.

Serum concentrations of total fatty acids are not different in children with a poor or a good nutritional status.[51] However, dienoic tetraenoic and hexaenoic acid levels are significantly reduced in the malnourished, while trienoic acid concentration is significantly higher. The significance of this is as yet unknown.

Fat balance studies in severely malnourished children show that they absorb only about 50 per cent of their fat intake.[52] The exact influence of these and other factors on lipid metabolism and maintenance of serum levels and partition in malnourished subjects remains to be known.

Evidence relating lipid and water metabolism in malnutrition is as yet scanty and conflicting. At the clinical level, while most observers describe polyuria as a characteristic feature of severe malnutrition,[53] others have found oliguria and delayed excretion of a water load, as well as presence of increased amounts of antidiuretic substances in the urine[54] and jugular blood.[55] An abnormal inactivation of antidiuretic substances in the damaged liver has been postulated for those cases.[54]

In experimental animals, marked water retention when small doses of pitressin are administered to rats fed low-protein alipotropic diets has been observed.[56] This has been corroborated in choline-deficient rats, which possess a diminished ability to inactivate pitressin,[57] and which can be restored to normal by vitamin B_{12} and aureomycin without influencing the fat content of the liver. Impaired water diuresis has also been found in mice fed protein-deficient diets,[58] with no correlation between the presence or intensity of fatty changes in the liver and the response to water administration. On the other hand, development of edema in weanling rats fed a protein-deficient diet can be prevented by lipotropic substances,[59] and actually a deficiency of them has been suggested as a possible etiologic factor in malnourished children with edema of the "kwashiorkor"

type. This could be compatible with the previously discussed association of clinical pitting edema, persistence of fat under the skin and intense fatty infiltration in the liver, and points to the eventual need of further evaluation of the role of these factors in infantile malnutrition.

The treatment of fatty liver is undoubtedly that of the malnourished child who bears it. Once an adequate diet is taken in, the liver will recover together with the remainder of the organism, showing the already described features of the nutritional recovery syndrome.[6]

Lipotropic agents have been tried by several groups, with the aim of further speeding recovery. Satisfactory results originally obtained with whole stomach extracts[60] need further confirmation. Acceleration of fat removal from the liver has been reported with the use of choline and lipocaic,[61] especially in cases with the most intense fatty infiltration. Since final recovery seems to be the same with or without such a treatment, the significance of this procedure cannot be assessed at the present time.

REFERENCES

1. Waterlow, J. C.: *Fatty Liver Disease in Infants in the British West Indies.* Spec. Rep. No. 263, Med. Res. Council, His Majesty's Stat. Off., London, 1948.
2. Waterlow, J. C.: in *Protein Malnutrition.* Proc. of a Conf. in Jamaica, 1953, FAO, Rome, 1955, p. 16.
3. Gillman, J. and Gillman, T.: *Perspectives in Human Malnutrition.* Grune & Stratton, New York, 1951.
4. Davidson, C. S. and Gabuzda, G. J.: Nutrition and disease of the liver. *New England J. Med.* 243: 779, 1950.
5. Trowell, H. C., Davies, J. N. P., and Dean, R. F. A.: *Kwashiorkor.* E. Arnold Ltd., London, 1954.
6. Gómez, F., Ramos-Galván, R., and Cravioto-Muñoz, J.: Nutritional recovery syndrome. Preliminary report. *Pediatrics* 10: 513, 1952.
7. Ambrosius, K. and Esparza, H.: Alteraciones hepáticas en la desnutrición infantil. *Bol. méd. Hosp. inf.* 13: 345, 1956.
8. Davies, J. N. P.: The essential pathology of kwashiorkor. *Lancet* 1: 317, 1948.
9. Meneghello, J., Niemeyer, H., and Espinosa, J.: Liver steatosis in undernourished Chilean children. I. Its evolution as followed by serial puncture biopsies. *Am. J. Dis. Child.* 80: 889, 1950.
10. Davies, J. N. P.: Kwashiorkor. *Tr. 9th Conference on Liver Injury.* Josiah Macy, Jr. Foundation, New York, 1951.
11. Bras, G. and Clearkin, K. P.: Histopathology of the pancreas in Jamaican infants and children. *Doc. Med. Geog. Trop.* 6: 327, 1954.
12. Bras, G., Waterlow, J. C., and DePass, E.: Further observations on the liver, pancreas, and kidney in malnourished infants and children. I. The relation of certain histopathological changes in the liver, pancreas and kidney. *J. Trop. Ped.* 2: 147, 1956.
13. Waterlow, J. C., Bras, G., and DePass, E.: Further observations in the liver, pancreas and kidney in malnourished infants and children. II. The gross composition of the liver. *J. Trop. Ped.* 2: 189, 1957.
14. Waterlow, J. C.: The protein content of liver and muscle as a measure of protein deficiency in human subjects. *West Ind. Med. J.* 5: 167, 1956.
15. Gómez, F., Ramos-Galván, R., Frenk, S., Cravioto, J., Chávez, R., and Vázquez, J.: Mortality in second and third degree malnutrition. *J. Trop. Ped.* 2: 77, 1956.
16. Bras, G.: in *Protein Malnutrition.* Proc. of a Conf. in Jamaica 1953, FAO, Rome, 1955, p. 94.
17. Frenk, S., Metcoff, J., Gómez, F., Ramos-Galván, R., Cravioto, J., and Antonowicz, I.: Intracellular composition and homeostatic mechanisms in severe chronic infantile malnutrition. II. Composition of tissues. *Pediatrics* 20: 105, 1957.
18. Addis, T., Poo, L. J., and Lew, W.: The quantities of protein lost by the various organs and tissues of the body during a fast. *J. Biol. Chem.* 115: 111, 1936.
19. Waterlow, J. C. and Weisz, T.: The fat, protein and nucleic acid content of the liver in malnourished human infants. *J. Clin. Investigation* 35: 346, 1956.
20. Copoletta, J. M. and Wolbach, S. B.: Body length and normal weights of more important vital organs between birth and twelve years of age. *Am. J. Path.* 9: 55, 1933.
21. Gómez, F., Ramos-Galván, R., Bienvenu, B., and Cravioto-Muñoz, J.: Estudios sobre el niño desnutrido. IV. Pruebas de funcionamiento hepatico en el preescolar sano y en el desnutrido, en el niño pelagroso y en el infectado. *Bol. méd. Hosp. inf.* 7: 485, 1950.
22. Niemeyer, H. and Meneghello, J.: Liver steatosis in undernourished Chilean children. II. Study of some liver function tests. *Am. J. Dis. Child.* 80: 898, 1950.
23. Kinnear, A. A. and Pretorius, P. J.: Liver function in kwashiorkor. *Brit. M. J.* 1: 1528, 1956.
24. Kinnear, A. A. and Pretorius, P. J.: Influence

of high-protein diets on liver-function tests in Kwashiorkor. *Brit. M. J.* 2: 389, 1957.

25. DEAN, R. F. A. and SCHWARTZ, R.: The serum chemistry in uncomplicated kwashiorkor. *Brit. J. Nutrition* 7: 131, 1953.
26. GÓMEZ, F., RAMOS-GALVÁN, R., CRAVIOTO, J., and FRENK, S.: El síndrome de recuperación nutricional en niños con desnutricion crónica avanzada que no presentaban edema clínicamente demonstrable ni lesiones dérmicas. To be published.
27. CRAVIOTO, J.: Proteins in malnutrition. *Mod. Prob. Paed.* 2: 169, 1957.
28. GITLIN, D., CRAVIOTO, J., FRENK, S., LOPEZ-MONTAÑO, E., RAMOS-GALVÁN, R., GÓMEZ, F., and JANEWAY, C. A.: Albumin metabolism in children with protein malnutrition. *J. Clin. Investigation* (in press).
29. VORHAUS, L. J. and KARK, R. M.: Serum cholinesterase in health and disease. *Am. J. Med.* 14: 707, 1953.
30. HUTCHISON, A. O., MCCANCE, R. A., and WIDDOWNSON, E. M.: Serum cholinesterases, in *Studies of Undernutrition*. Wuppertal 1946–49. Spec. Rep. No. 275, His Majesty's Stat. Off., London, 1951, p. 216.
31. GÓMEZ, F., RAMOS-GALVÁN, R., CRAVIOTO, J., and CHAVEZ, R.: Blood cholinesterases in chronic infantile malnutrition. In preparation.
32. HAWKINS, R. D. and NISHIKAWARA, M. T.: Effect of ingestion of hypolipotropic diet on level of pseudocholinesterase in plasma of male rats. *Biochem. J.* 48: 276, 1951.
33. BERRY, W. T. C., COWIN, P. J., and DAVIES, D. R.: A relationship between body fat and plasma cholinesterase. *Brit. J. Nutrition* 8: 79, 1954.
34. WATERLOW, J.: Liver choline-esterase in malnourished infants. *Lancet* 1: 908, 1950.
35. WATERLOW, J. C. and PATRICK, S. J.: Enzyme activity in fatty livers in human infants. *Ann. New York Acad. Sc.* 57: 750, 1954.
36. SRIRAMACHARI, S. and RAMALINGASWAMI, V.: Quoted by TROWEL, H. C., DAVIES, J. N. P., and DEAN, R. F. A.: in *Kwashiorkor*. E. Arnold, Ltd., London, 1954, p. 170.
37. GÓMEZ, F., RAMOS-GALVÁN, R., CRAVIOTO, J., FRENK, S., and BOLOK, R.: Los niveles séricos de calcio, fosforo y fosfatasa en preescolares desnutridos y en su recuperación. *Bol. méd. Hosp. inf.* 13: 865, 1956.
38. SCHWARTZ, R.: Alkaline phosphatase activity of the serum in kwashiorkor. *J. Clin. Path.* 9: 333, 1956.
39. BROCK, J. F., HANSEN, J. D. L., HOWE, E. E., PRETORIUS, P. J., DAVEL, J. G. A., and HENDRICKSE, R. G.: Kwashiorkor and protein malnutrition. A dietary therapeutic trial. *Lancet* 2: 355, 1955.
40. CHEUNG, M. W., BOWLER, D. I., NORTON, P. M., SNYDERMAN, S. E., and HOLT, L. E., JR.: Observations on amino acid metabolism in kwashiorkor. A preliminary report. *J. Trop. Ped.* 1: 141, 1955.
41. HOLT, L. E., JR.: in *Human Protein Requirements and Their Fulfilment in Practice*. Proc. of a Conf. in Princeton, FAO, Rome, 1957, p. 38.
42. ABALLI, A. J.: Las hipoglicemias en el niño desnutrido. *Bol. méd. Hosp. inf.* 13: 63, 1956.
43. HOLMES, E. G. and TROWELL, H. C.: Formation of hepatic glycogen in normal Africans and in those suffering from malignant malnutrition. *Lancet* 1: 395, 1948.
44. GÓMEZ, F., RAMOS-GALVÁN, R., CRAVIOTO, J., and FRENK, S.: Malnutrition in infancy and childhood, with special reference to kwashiorkor. *Advances Pediat.* 7: 131, 1955.
45. GÓMEZ, F., RAMOS-GALVÁN, R., CRAVIOTO, J., DELPINO, R., and FRENK, S.: Glucose tolerance in infantile malnutrition. In preparation.
46. WATERLOW, J. C.: Personal communication.
47. CARVALHO, M., PINTO, A. G., SCHMIDT, M. M., POTSCH, N., and COSTA, N.: Distrofia pluricarencial hidropigenica. *J. Pediat.* (Brasil) 11: 395, 1945.
48. GÓMEZ, F., RAMOS-GALVÁN, R., GALVÁN, R., CRAVIOTO, J., BURGOS, G., and FRENK, S.: Lipids and their fractions in blood serum of children with chronic severe infantile malnutrition and during their recovery. In preparation.
49. MANN, G. V., MUÑOZ, J. A., and SCRIMSHAW, N. S.: The serum lipid levels of Central Americans compared with those of North American adults. *Fed. Proc.* 13: 467, 1957.
50. JENCKS, W. P., HYATT, R. H., JETTON, M. R., MATTINGLY, R. W., and DURRUM, E. L.: A study of serum lipoproteins in normal and atherosclerotic patients by paper electrophoretic techniques. *J. Clin. Investigation* 35: 980, 1956.
51. HANSEN, A. W. and WIESE, H. F.: Essential fatty acids and human nutrition. II. Serum levels for unsaturated fatty acids in poorly-nourished infants and children. *J. Nutrition* 52: 367, 1954.
52. GÓMEZ, F., RAMOS-GALVÁN, R., CRAVIOTO, J., FRENK, S., VÁSQUEZ, J., and DE LA PÉNA, C.: Fat absorption in chronic severe malnutrition in children. *Lancet* 2: 121, 1956.
53. GÓMEZ, F., RAMOS-GALVÁN, R., CRAVIOTO, J., FRENK, S., JANEWAY, C. A., GAMBLE, J. L., and METCOFF, J.: Intracellular composition and homeostatic mechanisms in severe chronic infantile malnutrition. I. General considerations. *Pediatrics* 20: 101, 1957.
54. GOPALAN, C.: in *Protein Malnutrition*. Proc. of a Conf. in Jamaica, 1953, FAO, Rome, 1955, p. 37.
55. HELLER, H.: The antidiuretic potency of plasma in clinical conditions of protein deficiency. *Proc. XIX Internat. Physiol. Cong.* 1953, p. 449.

56. LLOYD, C. W.: Some clinical aspects of adrenal cortical and fluid metabolism. *Rec. Prog. Horm. Res.* 7: 469, 1952.
57. GUGGENHEIM, K. and DIAMANT, E. J.: Effect of riboflavin and choline deficiencies on water metabolism in rats. *J. Nutrition* 57: 249, 1955.
58. HELLER, H. and BLACKMORE, D. W.: Absence of correlation between fatty changes in the liver and impairment of water diuresis in protein deficient mice. *Brit. J. Nutrition* 7: 349, 1953.
59. ALEXANDER, H. D. and SAUBERLICH, H. E.: The influence of lipotropic factors on the prevention of nutritional edema in the rat. *J. Nutrition* 61: 329, 1957.
60. GILLMAN, T., GILLMAN, J., INGLIS, J., FRIEDLANDER, L., and HAMMAR, E.: The substitution of whole stomach extract for vitamins in the treament of malignant infantile pellagra. *Nature* 154: 210, 1944.
61. MENEGHELLO, J. and NIEMEYER, H.: Liver steatosis in undernourished Chilean children. III. Evaluation of choline treatment with repeated liver biopsies. *Am. J. Dis. Child.* 80: 905, 1950.

DISCUSSION

Dr. S. Stevenson (University of Pittsburgh, Pittsburgh, Pa.): This paper fascinates me because I have been intensely interested in this disease "kwashiorkor." I saw a fair amount of it in Egypt and in South America. I have never seen a typical case in Pittsburgh although *malnutrition*, I am quite sure, is the commonest cause of growth failure in children in the United States and is certainly the most widespread disease of children in the world, along with malaria, which may cause it. In this country, malnutrition is not usually caused by dietary lack but by disease, particularly infections. I think Dr. Frenk would agree that malnutrition is a disease in children whose etiologies may be legion, but whose end results are similar, but of varying severity.

Dr. Frenk, as a pediatrician, I want to say how long I have admired what you and your group are doing in the way of fundamental research into the effects and causes of malnutrition in childhood. I have also admired your complete refusal to relegate kwashiorkor to the realm of an exotic African disease and your classification of it under the term, as I think you once called it, "third degree malnutrition."

The commonest cause of severe malnutrition in Pittsburgh children—in this country, I suspect—is cystic fibrosis of the pancreas. This disease combines inability to digest and assimilate foodstuffs with chronic and severe infection. It is fairly common since it occurs in roughly 2 out of 1,000 live births in this country. We cannot cure the disease at present. We can treat the infection which accompanies it with antibiotics, and we do this fairly well. We can try to do something about the nutritional state of the child, and this we do not do very well.

Although cystic fibrosis causes severe malnutrition, which resembles the type Dr. Frenk sees on the Mexican plateau, there are some baffling differences between this state and the picture seen in kwashiorkor. Perhaps they are only in degree of severity; perhaps they are racial differences. I should like to ask about them.

In kwashiorkor, which has been translated "boy with red hair," you all know about the changes in the hair, which may fade to the point where it is almost gray or white. You all know about the skin changes. In severe malnutrition and cystic fibrosis, we do not find these hair and skin changes even though the children are terminally marasmic. I wonder if the hair changes have something to do with the inheritance of the Negro children in Uganda and Africa or whether they are due to a lack of phenylalanine.

I am intrigued with the mental changes of children with kwashiorkor—apathy, listlessness, almost dementia, in advanced kwashiorkor. I am intrigued because of phenylpyruvic oligophrenia, a disease we see in this country, where the child, usually a blond, is unable to metabolize phenylalanine properly and becomes demented and mentally deficient. Children with advanced cystic fibrosis and malnutrition are not apathetic and listless unless they are quite sick with secondary infection but are notably cheerful and bright.

I should like to ask you about the edema which is so universal in kwashiorkor and which we almost never see in cystic fibrosis. As a matter of fact, the serum albumin is not as depressed in cystic fibrosis as it is in kwashiorkor, and I wonder if the absence of edema in our children is because their serum albumin is not as depressed because there is less liver damage, or because they have almost no subcutaneous fat.

When you talk about the correlation between subcutaneous fat and edema in the kwashiorkor children, it makes me think of the old *Mehlnährshaden* the Germans described so well, which occurred in children on high carbohydrate, condensed milk diets in the old days.

We find, fairly routinely at postmortem, fatty livers in our malnourished children with cystic fibrosis. Although not always as severe as in the kwashiorkor babies, it is the same type, starting from the periphery of the lobule and spreading centrally.

I wonder about two more things. First, are the sudden deaths which are seen in kwashiorkor, and also in cystic fibrosis, possible hepatic deaths? Second, I am thoroughly confused about choline after the earlier discussions this morning and after what Dr. Frenk has said. We don't give our cystics choline in dietary therapy. I wonder if we should.

One last thing. You talked about the recovery syndrome, when the liver becomes large, and I know that you have shown in other studies that it enlarges partly because the cells become larger. However, by treatment, you increase the caloric (and carbohydrate) intake and presumably up the metabolic rate and I wonder if some of this liver enlargement may be due to congestive cardiac failure due to beriberi.

Dr. Robert Kark (University of Illinois, Chicago): I wonder if Dr. Frenk would dwell again on skin chemistry. I am not quite sure what he is measuring. Is it skin or subcutaneous tissue or both together?

The second point concerns the fat excreted by the children. This summer in North Africa I saw a number of children of the kind he described, but in addition there were a fair number who had typical celiac disease. I wonder whether he found neutral fat in the stools of the marasmic children or whether it was primarily fatty acids? Does he see celiac disease in Mexico?

Dr. Labecki: Do the increased proportions of beta-lipoprotein in children with kwashiorkor decrease with clinical improvement? They are definitely increased by adult standards in the tables you showed.

Dr. E. E. Howe (Merck Institute for Therapeutic Research, Rahway, N. J.): I have had the good fortune to work with Dr. Brock and Dr. Hansen on the use of amino acids and can partially answer one of the questions that Dr. Frenk raised about continuing studies with amino acids. Of course, it is rather expensive to continue these studies past 30 days with the crystalline amino acids. If, however, these children, after the initiation of cures are placed back on maize plus a little additional glycine, they will continue to improve.

Dr. Wilgram: In extension of Dr. Frenk's presentation I would like to mention that over the last few years we have conducted an experiment, a well controlled study of monkeys on a borderline protein-deficient diet, which is also definitely deficient in lipotropic substances such as choline. Those animals just barely maintain their weight. They do not lose, but they do not gain, either. Yet the livers that we see resemble exactly what you have shown here, Dr. Frenk, in children with kwashiorkor.

If I may repeat this briefly, we see a periportal fat accumulation of the type where the fat appears in big confluent droplets quite different from the protein-deficient liver which we see in the rats.

You mentioned that the effect of choline is apparently beneficial in your humans. I might perhaps offer the personal opinion that under those circumstances choline may be methionine-sparing. By adding choline to such a diet one may give the child, in your case, or the monkey in our case, help in preserving its methionine reserves.

Dr. Frenk (closing remarks): I thank you all very much for your comments. Regarding the lack of changes in hair color in cystic fibrosis of the pancreas, as compared to those found in chronic severe malnutrition, I do not think that this is related to racial characteristics of African children. The babies of the central part of Mexico, with no African blood, show the same hair changes. Some of them become not only reddish but blond or even white-haired. You may see in the hair of these children the so-called "sign of the flag"; revealing all of the different colors which the hair has passed through during the illness.

We probably have as much cystic fibrosis of the pancreas as you have in the United States, but isolated cases may get lost in the overwhelming numbers of severely malnourished children. If we had a whole hospital just for this disease, we could fill it up in five minutes.

Whether the apathy of these children may be related to the same hypothetical factors which cause the hair changes, I am not yet prepared to say. Actually, as you know perfectly well, these mental changes disappear very quickly when recovery starts.

Perhaps the lack of pitting edema in cases of cystic pancreatic fibrosis is due to the lack of subcutaneous fat. I would not really be able to answer this. On the other hand, as you say, Professor Stevenson, everybody agrees now on the fact that *Mehlnährschaden* and kwashiorkor are one and the same disease. But marasmus, which clinically looks so different, should also be considered, as the other extreme of the same process.

Regarding sudden death in these children, we see it quite frequently. Several people, especially in Cuba, believe that it may be related to an abnormal handling of carbohydrates. Actually, when these babies die, they may show a profound hypoglycemia (10 to 15 mg per 100 ml) immediately before death.

I do not think that a thiamine deficiency is the cause of the nutritional recovery syndrome. The children who show it look so well, are running around the ward and behaving so normally or supranormally, that one would rather doubt that they are in cardiac insufficiency. We sometimes see congestive cardiac insufficiency in older children, but not in babies between two and four years of age, which is the group which characteristically shows this syndrome.

It probably is the big, clear, granular hepatic cell which I showed you, which makes the liver enlarge during recovery, just as happens in von Gierke's disease. Why do these children get ascites at the same time? I really don't know and I don't think anybody knows.

Regarding the very good question of Dr. Kark, our "skin" biopsies include skin plus subcutaneous tissue. The way we do the biopsy is to go straight down to the fascia and lift the whole flap. So we obtain what many people believe works as a functional unit.

Our children do have chemical steatorrhea. They don't have clinical steatorrhea unless you give them a high-fat diet. You saw that the absorption was only about 50 per cent of the intake. If you give 10 g of fat, 5 g will come out in the stool; if you give 50 g, 25 will come out, and you will see clinical steatorrhea. But I have not believed that any of these children could be confused with those having celiac disease, which in Mexico presents exactly as celiac disease does in other parts of the world.

Serum β-lipoproteins show changes parallel to those of total lipids; increasing or decreasing according to the evolution of the latter in the serum of a malnourished child during recovery. Their proportion

i.e. $\beta:\alpha$ ratio decreases with recovery. Further work on this is needed.

I am very grateful for the information on the synthetic amino acid diets. Dr. Howe's remarks more or less agree with our findings showing a good initial response when glycine is added to a poor diet.

I heard with great interest what Dr. Wilgram told us about the distribution of fat in the livers of monkeys. Perhaps the monkey when subjected to deficient diets gets the same disease as our children.

Dr. Kark: Dr. Frenk, you did not tell us whether the steatorrhea represented neutral fat or fatty acids.

Dr. Frenk: Both neutral fat and fatty acids are present.

The Effect of Low-Protein Diets Upon Serum Cholesterol in Man

ROBERT E. OLSON, PH.D., M.D.,* JOHN W. VESTER, M.D.,** DEHA GURSEY, M.D.,†
NORMAN DAVIS, M.D.,‡ AND DORIS LONGMAN, M.S.§

IT IS AN interesting fact that adult populations which subsist upon diets which cause kwashiorkor in infants have relative freedom from atherosclerosis. Kwashiorkor and atherosclerosis are, in a sense, reciprocal disorders. In countries where the food supply is good and the nutrients supplied in abundance, the infants do well and the adults do poorly (*vis a vis* degenerative disease); conversely in countries where the food supply is poor and the nutrients marginal or even limiting, the infants do poorly and the adults appear to be less susceptible to vascular and other degenerative diseases. Even in our country this biologic spectrum is represented at one end, the pole of undernourishment, by the chronic alcoholic, and at the other end, the pole of *luxus* nutrition, by our obese population which appears to be susceptible not only to atherosclerosis[1] but also to diabetes, hypertension, and biliary disorders.

From the Department of Biochemistry and Nutrition, Graduate School of Public Health, the Department of Medicine, School of Medicine, University of Pittsburgh, and the Medical Service, St. Margaret's Memorial Hospital, Pittsburgh, Pennsylvania.

* Professor and Head of the Dept. of Biochemistry and Nutrition; ** Assistant Professor of Biochemistry and Nutrition; † Research Fellow in Nutrition; ‡ Clinical Associate in Nutrition; § Research Associate in Nutrition; Graduate School of Public Health, University of Pittsburgh.

Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc., New York, New York.

Supported in part by grants-in-aid from the National Vitamin Foundation, New York, the Nutrition Foundation, New York, and the Division of Alcoholic Studies and Rehabilitation, Department of Health, Commonwealth of Pennsylvania, Harrisburg, Pennsylvania.

The physiologic events responsible for the relative incidence of atherosclerosis along the whole expanse of this biologic gradient are, of course, the product of many variables as diets change from "bad" to "good." We should like to propose, however, that a significant factor in the adjustment to diet by these populations is the endocrine function of the liver as it pertains to the secretion of β-lipoproteins. This may be looked upon as a specific liver function which is sensitive to states of undernutrition and overnutrition with regard not only to calories and to fat, but also to protein and possibly a number of lipotropic factors.

Evidence supporting this view will be presented under the four general headings of: the pathogenesis of atherosclerosis; the epidemiologic data relating degenerative heart disease to diet; the effect of hypolipotropic diets upon the serum lipids and lipoproteins of the rat; and the effect of hypolipotropic diets upon the serum lipids and lipoproteins of man. A preliminary report of this work has been presented.[2]

PATHOGENESIS OF ATHEROSCLEROSIS

It is beyond the scope of this paper to attempt to consider this extraordinarily broad subject in but the sketchiest outlines. It seems reasonably clear, however, that atherosclerosis is a disease of multiple etiology resulting from a complex interaction between organism and environment.[3] The "agent" of the disease, furthermore, appears to arise within the host as a result of this interaction. Instead of being an independent variable, as is the case in diseases of infection, addiction, or nutritional deficiency, the "agent" of atherosclerosis is a dependent variable developing

from many possible host-environment adjustments. The fact that it is "endogenous," furthermore, complicates the making of a distinction between agent, host and environment in a classic ecologic framework. Nevertheless, it seems to us that this viewpoint is essential in further studies designed to delineate with more certainty the "agents" and the host responses which lead to atheromatosis. Some of the agents which have been proposed as etiologic in atherosclerosis have included cholesterol,[4] S_{f12-20} β-lipoproteins,[5] minute thrombi,[6,7] altered internal elastic membrane of the artery,[8] and proliferating intima.[9] Some of the host factors which may influence the genesis of the "agent" include genetic endowment, race, age, sex, endocrine balance, and psychic state. Some of the equally pertinent environmental factors include diet, drugs, exercise, occupation and sociocultural status.

Most modern investigators agree with Virchow[10] that the pathogenesis of atherosclerosis involves an alteration in the normal processes of filtration and resorption of plasma through the intima and the subadjacent media of the involved artery. Page[11] has recently pointed out that retention of lipids in the subintimal space could result from alteration in the nature or amount of the β-lipoproteins, change in the integrity of the intima or of the internal elastic membrane, or modification of the metabolism of the arterial wall. The reasonableness of a hypothesis of multiple etiology is immediately clear from such an analysis of the probable pathogenesis of the lesion.

It is apparent that both *humoral* and *local* factors are involved. Under humoral factors one may list the β-lipoproteins, the proteins involved in the coagulation schema[12] and heparinoid substances involved in clearing. Under local factors one can identify (a) hemodynamic factors relating to blood flow, turbulence and pressure, (b) metabolic factors intrinsic in arterial tissue which control its respiration, lipogenesis, and lipoprotein transformation, (c) structural factors relating to anatomic differentiation of intima, internal elastic membrane, and other components of the arterial wall, and (d) traumatic factors relating to injury, ulceration, and repair.

Since the organ most concerned with the elaboration of the humoral factors is the liver, it seems reasonable to assume that this organ may play a decisive role in the pathogenesis of atherosclerosis. The evidence supporting this postulate is summarized below.

The level of serum lipids in the mammal under various conditions of diet and metabolic state reflect principally the interaction of three organs, the intestine, the liver, and the fat depots in accomplishing the addition of lipid to or removal of lipid from the plasma. A simplified schema of lipid transport among gut, liver, and depots emphasizing the central role of the liver is shown in Figure 1. Fat and cholesterol

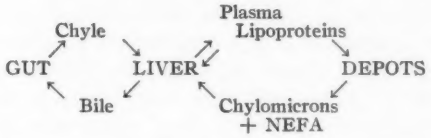


Fig. 1. Schematic diagram of lipid transport in the mammal. (NEFA represents non-esterified fatty acids.)

absorbed from the gut are transported from the lacteal via the thoracic lymph as chylomicron lipid to the general circulation.[13,14] These chylomicrons are then rapidly cleared from the blood. The tissues which participate in this clearing have not all been identified, but it has been shown that elements of the reticuloendothelial system, particularly the Kupffer cells of the liver are very active in this process.[15] The fatty acids from the cleared triglycerides appear rapidly in the nonesterified fatty acid fraction (NEFA) which is taken up by many tissues. including the liver. The cleared cholesterol is apparently transferred directly from the Kupffer cell to the hepatic parenchymal cell where it becomes available for excretion in the bile (as bile or cholesterol) or secretion into the plasma as lipoprotein. In man this transfer mechanism for dietary cholesterol results in a lag of three days in the attainment of maximum specific radioactivity in the plasma cholesterol after feeding cholesterol-1-C^{14}.[16]

The formation of plasma lipoproteins is an important liver function and apparently the most important, if not the sole means by which

hepatic lipid, of either endogenous or exogenous origin, is transferred to the periphery. Although many tissues synthesize cholesterol, only the liver contributes this sterol to the plasma[17] and retrieves it again for ultimate catabolism. Plasma phospholipids, like plasma cholesterol, are both derived from and removed by the liver.[18] Hepatectomy, therefore, has relatively little effect upon the plasma concentrations of phospholipid and cholesterol,[19] although it abolishes turnover. It would appear, therefore, that plasma phospholipid and cholesterol, together with the protein moiety of the plasma lipoprotein serve as the vehicle for lipid transport from liver to periphery (particularly the depots) without actively contributing to it. In contrast to phospholipid and cholesterol ester, lipoprotein-bound triglyceride appears to be the chief medium of lipid transfer from the liver to the depots. The rapid turnover time of plasma triglyceride fatty acids as compared with phospholipid or cholesterol ester fatty acids supports this view.[20,21] Furthermore, adipose tissue is a rich source of lipoprotein lipase[22] which could act to liberate fatty acids from plasma lipoprotein for assimilation in the depots.

Whereas the adipose depots take up carbohydrate and lipoprotein lipid during periods of feeding, they discharge lipid in the form of nonesterified fatty acids (bound to albumin) and chylomicrons during periods of fasting.[23,24] This phenomenon is intensified in conditions of starvation, diabetes mellitus, and anterior pituitary excess. In these instances, however, the plasma triglyceride is elevated out of proportion to cholesterol and phospholipid and the plasma is lactescent. Since the liver is the only source of plasma cholesterol and phospholipid, it seems certain the hypercholesterolemia and the hyperphospholipidemia which accompany the lipemia in these instances is part of the hepatic response to increased mobilization of triglycerides from the depots.[25–27] The persistence of postprandial chylomicronemia (high-fat feeding, diabetes, obesity, idiopathic hyperlipemia) appears to result in an increased hepatic elaboration of β-lipoproteins with consequent elevation of plasma phospholipid and cholesterol. This, in turn, may contribute to the progression of atherosclerosis. It seems clear, from this brief and elementary consideration of known mechanisms of fat transport, that dietary factors which modified any of the rates pictured in Figure 1 could affect the level of lipid in any or all of the compartments shown. Evidence is available to show that caloric balance, kind (degree of unsaturation) and amount of dietary fat, and the kind and amount of dietary protein influence serum lipid levels in man. Other dietary factors, of importance in fat transport in animals, may also be important in man, although the data are not yet compelling.

In relating any of the above constituents of the diet to the clinical events signifying atherosclerosis we must be fully aware of the extraordinary number of extradietary factors which are involved in the final outcome. One can, of course, outline a working hypothesis relating diet to coronary artery thrombosis by way of liver function, serum lipid changes, atheroma formation, and final occlusion, as shown in Figure 2.

One must be careful to insert a number of vectors representing other forces than those in the main stream of diagram to emphasize the uncertainty of the progression in given individuals. All of the pertinent humoral and local factors operating at the vascular level, the basic "set" of the liver in elaborating β-lipoproteins (possibly conditioned by genetic factors) and its responsiveness to given diet composition, the likelihood of the lesions being in particularly vulnerable sites, if formed, and the chance of their precipitating clinical disease, are factors which make the *a priori* generalizations

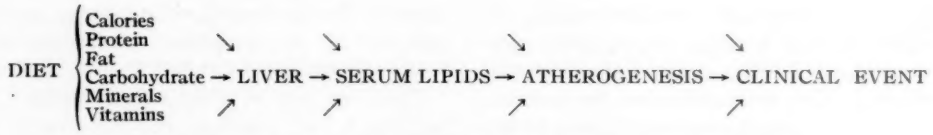


Fig. 2. The role of diet in the multifactorial pathogenesis of atherosclerosis.

about the effect of diet composition upon the incidence of clinical "events" essentially worthless. In large groups, with different diet patterns, some of the pertinent variables other than diet tend to be randomized, but even in such groups extreme caution should be exercised in drawing conclusions from superficial associations.

EPIDEMIOLOGIC STUDIES OF DIET AND HEART DISEASE

The epidemiologist attempts to relate given environmental factors to disease. Keys and his associates[28,29] have carried out studies of the relation of dietary fat intake to coronary artery disease in many parts of the world. He has concluded that a strong association exists between the incidence of coronary artery disease in many populations and the magnitude of the habitual intake of dietary fat. In fact, Keys plotted the incidence of "degenerative heart disease" in six countries vs. per cent of dietary calories derived from fat[28] and obtained a convincing parabola which gives a linear plot on semilogarithmic coordinates. In fact, it is a very good "dose-response" curve in the pharmacologic sense. It has been pointed out recently, however, by Yerushalmy and Hilleboe[30] that when the available data from 22 countries are studied, "the tests for specificity show that the association (between dietary fat and heart disease mortality) lacks validity." Both these authors[30] and Yudkin[31] found a stronger association between dietary animal protein and mortality from coronary artery disease than between dietary fat and that mortality. We, independently, had noted that the plot of animal protein intake vs. mortality from degenerative heart disease for the six countries Keys had initially described[20] gave the same shaped curve as for fat.[32] This, of course, does not prove that the intake of dietary fat has no bearing on the pathogenesis of coronary artery disease any more than it proves that the intake of animal protein is the critical factor. Epidemiologic associations are never independently conclusive, but require additional clinical investigation for validation. Such clinical studies have shown that altering the amount and kind of dietary fat does influence serum lipid levels in man, although the extent to which such measures influence the course of clinical atherosclerosis is controversial at the present time.[33] We shall present evidence that the animal protein intake may also influence the serum cholesterol level in animals and man.

EFFECT OF HYPOLIPOTROPIC NUTRITION ON SERUM LIPIDS AND LIPOPROTEINS IN THE RAT

This study actually began 18 years ago when one of us (R.E.O.) was a graduate student in the laboratory of Dr. Wendell Griffith at St. Louis University. Dr. Griffith who is one of our essayists in this Symposium, had just described the syndrome of hemorrhagic renal degeneration as a manifestation of choline deficiency in the weanling rat[34] and suggested to me that I undertake an over-all study of lipid metabolism in the young choline-deficient rat. We observed that hypolipemia was a regular occurrence in the fed choline-deficient rat in association with fatty liver, but that fasting, or the inanition associated with the onset of the renal lesion, caused an increase in serum lipids and tended to obscure the effect. Unfortunately, the work was interrupted by the exigencies of the war and was not resumed in our laboratory until two years ago.

In these studies, young Sprague-Dawley male rats of the Holtzman strain weighing about 120 grams were fed experimental diets representing the permutations of low/high-methionine, low/high-choline, low/high-fat, low-high-unsaturated-fat and low/high-cholesterol. The level of dietary protein was 18 per cent by weight with soy protein (Drackett) providing the low methionine level (0.16 per cent) and casein providing the high methionine level (0.54 per cent). The total organic sulfur of these rations was kept constant at 0.2 per cent by varying the content of cystine. The range of the choline content was to 0–0.3 per cent, that of fat, 6–40 per cent, and that of cholesterol 0–1 per cent. Corn oil, butter, and lard were employed as sources of fat. The animals were fed these diets for two weeks and then sacrificed. Serum lipids and lipoproteins were determined on pooled samples of serum from three or more animals. The serum and liver lipids were determined by conventional methods as previously described[32]

and the serum lipoproteins by the method of Gofman.[35]

Typical results for groups of 8 to 12 rats are shown in Tables I and II. Data for rats fed the Purina chow stock ration are also included. The rat characteristically has lower levels of serum lipids than man[36] and an appreciably different profile of serum lipoproteins.[37] Generalized hypolipemia was found in all rats fed diets low in methionine and choline. Serum cholesterol, phospholipid, and triglyceride all declined and the low-density β-lipoproteins virtually disappeared. Elevation of the fat content of the soy protein or the change in its iodine number, in the absence of choline, did not influence the hypolipemia. The addition of choline prevented the hypolipemia, the hypo-β-lipoproteinemia, and resulted in slightly higher values for phospholipid, triglyceride, and high-density α-lipoproteins than found in animals on the stock ration. The substitution of casein for soy protein without added choline minimized the hypolipemia, and the hypo-β-lipoproteinemia although it did not completely abolish it. It is assumed that the extra methionine contributed its methyl group for choline synthesis, and this is borne out by the lesser fatty infiltration observed in the casein-fed rats. The less dramatic effect of low choline rations upon the serum lipoproteins of rats studied by Wilgram, Lewis, and Blumenstein[38] was probably due to the more generous amounts of methionine supplied by their ration. In low choline diets fortified with 1 per cent cholesterol plus 1 per cent taurocholate no hyperlipemia was noted unless choline was added to the ration,[39] an effect also noted by Ridout *et al.*[40] and by Wilgram, *et al.*[41]

Since conversion of dietary cholesterol to β-lipoprotein cholesterol involves intermediary transformation in the liver, the failure to see hypercholesterolemia after cholesterol feeding in the choline-deficient rat is not unexpected. This syndrome, i.e., hypolipemia in association with fatty liver, strongly suggests that fat-transport from liver to periphery is being retarded. Stetten and Salcedo[42] concluded on the basis of studies of the distribution of deuterium-labeled fatty acids synthesized from enriched body water in liver and adipose tissue that a defect in transport existed between the liver and the depots in the choline-deficient rat. Marked hypolipemia and hypocholesterolemia in association with fatty liver has also been seen in the choline-deficient puppy.[43] Although Zilversmit, Entenman, and Chaikoff[44] found no increase in the turnover of plasma phospholipids in the mildly choline-deficient dog given choline, no studies of the turnover of plasma-triglycerides or cholesterol were made and it is possible that the turnover of these fractions, particularly the triglyceride fraction which appears to be the "transport form" of lipid from liver to depots, was altered. Although the precise action of choline influencing the fat metabolism of the liver still remains an enigma, a role in stimulating lipoprotein formation for the transport of triglyceride to the periphery seems a strong possibility.

EFFECT OF HYPOLIPOTROPIC DIETS UPON THE SERUM LIPIDS AND LIPOPROTEINS IN MAN

The relevance of these studies in the rat to man is of paramount importance. One cannot transpose data from one species to another without direct evidence. In another primate, the Cebus monkey, Mann *et al.*[45] found that hypercholesterolemia from cholesterol feeding was not obtained unless choline was added to the diet. Furthermore, the rice diet, introduced by Kempner[46] for the treatment of hypertension in man is one of the most potent hypocholesterolemic rations known. It contains 25 g of protein (of which methionine is the limiting amino acid), 5 g of fat, and carbohydrate (from fruit and fruit juice) in quantity sufficient to maintain caloric balance. Although the hypocholesterolemic effect of this diet has been attributed to its low fat content by Keys *et al.*[47] the decreases in serum cholesterol which have been observed[48–50] are out of proportion to what has been observed on diets low in fat but replete with protein.[51]

In view of these considerations, we undertook a study of the effect of low-protein hypolipotropic nutrition upon the serum cholesterol of nine human subjects, five of whom were hypercholesterolemic. These subjects were hospitalized on a metabolic unit and fed a weighed control diet containing 100 g of protein, 80 g of

TABLE I

Effect of Dietary Choline, Methionine, and Fat Upon the Serum Lipids and Lipoproteins of Rats

Diet, %				Liver lipid, %	Serum lipids, mg/100 ml				Serum lipoproteins, mg/100 ml					
									High density (α)-S_6	Low density (β)				
Number	Lard	Methionine	Choline	Total	Cholesterol	Phospholipid	Triglyceride	Total		S_{f0-12}	S_{f12-20}	$S_{f20-100}$	$S_{f100-400}$	Total
Chow	—	—	—	4.1	90	139	30	311	125	38	0	37	14	89
1	6	0.16	0.0	30.1	49	95	7	174	90	2	0	2	0	4
2	6	0.16	0.3	5.9	87	188	94	410	150	28	0	62	74	164
3	40	0.16	0.0	35.6	50	126	8	207	147	0	0	0	0	0
4	40	0.16	0.3	6.2	93	184	86	406	195	37	0	38	13	88
5	40	0.54	0.0	22.9	88	165	12	306	120	22	0	18	15	65
6	40	0.54	0.3	5.8	90	166	54	352	158	38	0	28	8	74

TABLE II

Effect of Type of Fat on the Serum Lipids and Lipoproteins of Rats Fed Low-Methionine Diets with and without Choline

Diet, %			Liver lipid, %	Serum lipids, mg/100 ml				Serum lipoproteins, mg/100 ml					
								High density (α)-S_6	Low density (β)				
Number	Fat*	Choline	Total	Cholesterol	Phospholipid	Triglyceride	Total		S_{f0-12}	S_{f12-20}	$S_{f20-100}$	$S_{f100-400}$	Total
Chow	—	—	4.1	90	139	30	311	125	38	0	37	14	89
3	L	0.0	35.6	50	126	8	207	147	0	0	0	0	0
4	L	0.3	6.2	93	184	86	406	195	37	0	38	13	88
7	Bu	0.0	29.1	56	102	20	204	119	3	0	1	0	4
8	Bu	0.3	8.5	99	171	91	409	181	23	0	31	9	63
9	CO	0.0	26.5	59	104	28	220	142	2	0	0	0	2
10	CO	0.3	8.9	106	177	94	427	210	28	0	35	14	77

* Level of dietary fat in all experiments 3–10 was 40% by weight; L = lard; Bu = butterfat; CO = corn oil.

fat (*ca* 30 per cent cal) and 300 to 350 g of carbohydrate to supply about 2,400 cal for one to two weeks. The dietary fat was derived chiefly from animal sources and had an average iodine value of 42. The protein was derived mainly from animal sources and supplied the essential amino acids in amounts[52] of four to eight times the estimated human requirement.[53] At the end of the control period, the subjects were fed an isocaloric isofatty (including 50 g of butterfat) diet containing 25 g of vegetable protein derived from cereals, rice and legumes for one week. All essential amino acids except methionine were supplied by the low-protein diet in amounts which met Rose's tentative minimum requirement (Table III) and all other essential nutrients were given in adequate amount (partially supplied by a vitamin-mineral supplement) (Table IV). The low-protein diet contained between 0.18 and 0.22 g of choline whereas the control diet contained between 1.0 and 1.2 g of choline. At the end of the low-protein period, the subjects were again fed the control diet. Serum cholesterol was measured twice weekly. In several instances, nitrogen balances were measured.

TABLE III

Daily Intake of Essential Amino Acids and Choline by Experimental Subjects

Amino acid	Diet I (100 g protein)	Diet II (25 g protein)	Rose's minimal daily requirements
Arginine	5.19	1.06	—
Histidine	2.79	0.43	—
Isoleucine	5.66	0.92	0.70
Leucine	8.50	1.39	1.10
Lysine	7.15	0.90	0.80
Phenylalanine	4.54	0.93	1.10
Tyrosine	3.81	0.42	—
Threonine	4.29	0.70	0.50
Tryptophan	1.19	0.20	0.25
Valine	5.96	0.95	0.80
Methionine	2.32	0.30	1.10
Cystine	1.35	0.30	—
Choline	1.20	0.20	—

All subjects showed a decrease in serum cholesterol during the low-protein period which averaged 44 ± 4 (S. E. difference) mg per 100 ml as shown in Table V, and all values returned toward normal upon resumption of the control diet. The changes in body weight were nil during these relatively short term studies. One of the subjects, AK, was studied in considerably more detail and for a longer (10-week) period on the low-protein regimen. The changes in his body weight, serum lipids and serum β-lipoproteins are shown in Table VI. The changes are not as dramatic as those seen in the rat given hypolipotropic diets but they are identical in kind. Serum cholesterol and phospholipid and the serum lipoproteins reached a plateau in about two weeks and remained at that minimum point during the remainder of the low-protein feeding period. The degree of negative nitrogen balance changed throughout the low-protein period. During the first two weeks it averaged −0.85 g/day and for the re-

TABLE IV

Daily Intake of Essential Nutrients by Experimental Subjects

Diet	Calories	Protein *g*	Fat *g*	Carbohydrate *g*	Calcium *g*	Iron *mg*	Thiamine *mg*	Riboflavin *mg*	Niacin *mg*	Ascorbic acid *mg*
Control	2,400	100	80	320	1.40	16	1.2	2.6	15	90
Experimental	2,400	25	80	395	0.54	12	3.0	2.7	29	200

TABLE V

Effect of Low-Protein Diets Upon Serum Cholesterol in Man

Period	Patients No.	Diet				Body wt. *kg*	Serum cholesterol, mg/100 ml		
		Protein *g*	Fat *g*	Carbohydrate *g*	Calories *g*		Mean	Range	Change
Control	9	100	80	320	2,400	61.4	311	215–460	—
Experimental	9	25	80	395	2,400	61.2	267	160–420	−44 ± 4

maining period of eight weeks, it averaged −0.14 g/day. The degree of hypocholesterolemia did not parallel the degree of negative nitrogen balance and hence it seems unlikely that the effect upon serum lipids is due to a net catabolism of body protein. It seems more likely that the effect is due to an amino acid imbalance in which methionine and other lipotropic factors play a role. Following return of this subject to the control regimen, after ten weeks, his serum cholesterol and lipoproteins promptly increased to values equal to or greater than the previous control values.

No signs of liver dysfunction were noted throughout the study. There was no hepatic enlargement and all of the clinical laboratory tests of liver function (bromsulfalein retention, serum bilirubin, alkaline phosphatase, cephalin flocculation, albumin/globulin ratio, and prothrombin time) remained within normal limits. His liver was biopsied at two-week intervals and four of the sections taken at the beginning of the low-protein period, and at 2, 4, and 10 weeks are shown in Figure 3. Dr. John Kurtz, the pathologist at St. Margaret's Memorial Hospital, could see no significant change in these sections. The sudan-stained sections showed a small amount of finely divided fat in some of the liver cells at the outset of the study but there was no progression during the period of low-protein feeding. Kark, Horwitt, and Rothwell[54] fed somewhat more liberal low-protein diets (6.5 g N per day) to human subjects over a much longer period of time (365 to 458 days) and noted hepatomegaly, bromsulfalein retention and abnormalities in lactate and pyruvate clearance which were corrected by feeding protein supplements but not by separate lysine, methionine, or choline supplements. The difference is probably due to the time factor.

It is probable that methionine and/or choline play a role in the hypocholesterolemic response to low-protein feeding in man as they do in the rat. This is shown by a metabolic study of a mildly hypercholesterolemic but otherwise healthy middle-aged alcoholic female, FK, presented in Figure 4. During the first 25 days, the effect of the routine low-protein moderate fat diet described above is shown.

TABLE VI

Effect of a Low-Protein, Low-Choline Diet Upon Serum Lipids and Lipoproteins in Man*

Diet		Period		Serum lipids, mg/100 ml				Serum lipoproteins, mg/100 ml			
								Low density (β)			
Nature	Protein g	Weeks on diet	Body weight Kg	Cholesterol	Phospholipid	Triglyceride	Total	S_{f0-12}	S_{f12-20}	$S_{f20-100}$	$S_{f100-400}$
Control	100	2	62.2	258	293	70	743	409	22	79	17
Experimental	25	2	62.1	218	232	60	612	298	11	56	18
		4	60.6	213	223	22	558	324	10	35	8
		10	58.8	220	242	48	612	293	32	16	4
Control	100	1	59.2	287	346	236	994	403	30	118	43

* AK—male 41 years of age.

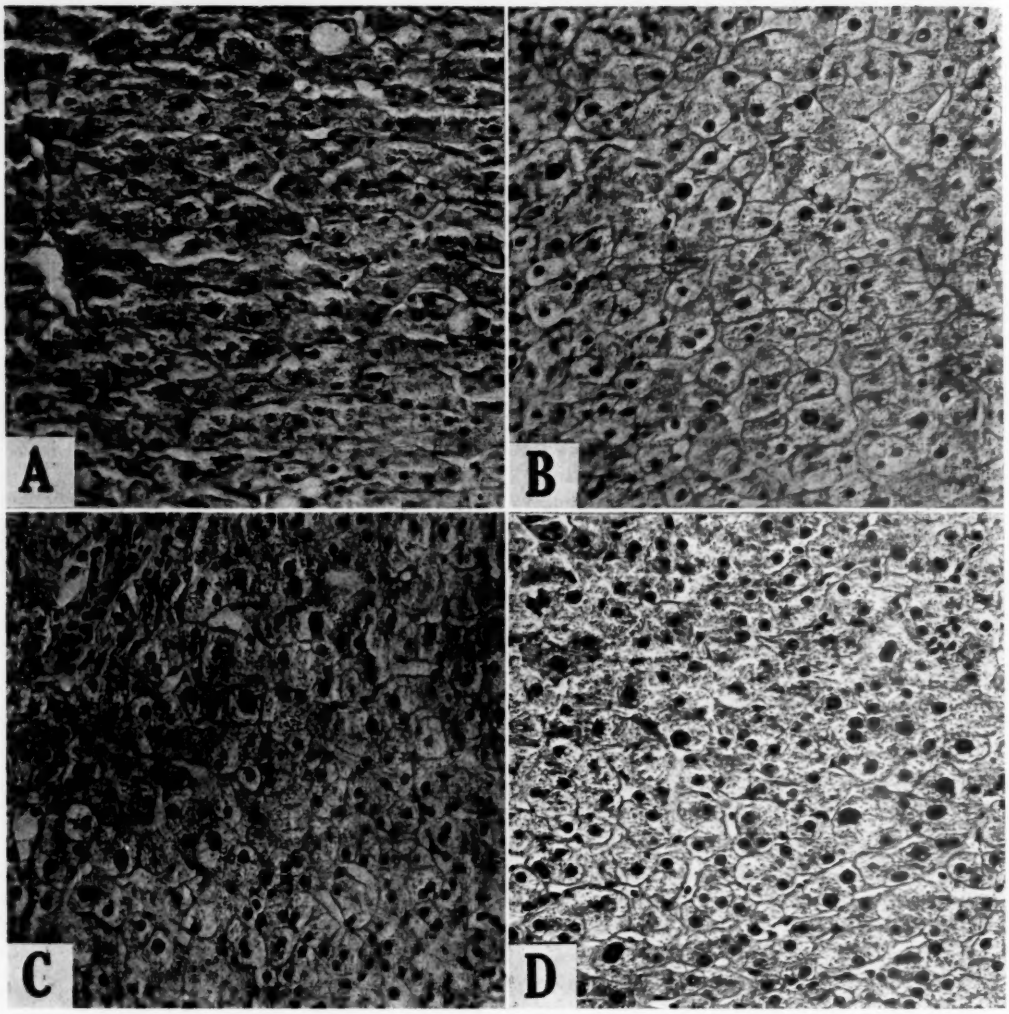


Fig. 3. Liver biopsy specimens obtained from patient AK during the low-protein study. (A) Liver tissue obtained while subject was fed the control diet containing 100 g of protein. Liver tissue obtained after intervals of feeding the 25 g protein diet are shown in (B) (2 weeks); (C) (4 weeks); and (D) (10 weeks). All sections show liver cells which are large, well-outlined, and polyhedral with light finely granulated cytoplasm. There are no vacuoles resembling those out of which fatty material has been dissolved. Some of the liver cells contain finely divided yellowish-brown pigment granules. All sections fixed in formalin and stained with haematoxylin and eosin (× 280).

Her hypocholesterolemic response over the one-week period was impressive and was abolished by refeeding the 100 g protein diet. She was then fed *ad libitum* for a week followed by the rice-fruit regimen, low in protein and fat. On the latter diet, her cholesterol came down approximately to that seen with low-protein feeding previously, and the pattern of change in nitrogen balance was essentially identical to that seen in the previous test. Butterfat was then added to the rice in amounts isocaloric with the fat fed during the control period and in this patient there was *no response* of the serum cholesterol to the addition of dietary fat.

A supplement of lipotropic factors (Methiscol®) supplying 2.5 g of choline dihydrogen citrate (equivalent to 0.87 g choline), 1.0 of DL-methionine, 0.75 g of inositol, 0.78 g of liver concentrate, and 18 μ of vitamin B_{12} was added and, without any other change in the diet, there was a prompt hypercholesterolemic response with restoration of the serum cholesterol to its previous "normal" value. Elevation of dietary protein to 100 g in the last period had no further effect upon the serum cholesterol value. Although the multiplicity of factors in the supplement makes interpretation difficult, it seems probable that methionine and/or choline were the active factors. Further studies of this kind are in progress with single lipotropic supplements to define clearly the nutrient responsible for the effect. Not all patients are totally unresponsive to the addition of butterfat to the rice diet, as was FK, but only slight to moderate rises are noted. In our experience addition of protein or lipotropic factors is essential to obtain a return of the serum cholesterol to the previous control value.

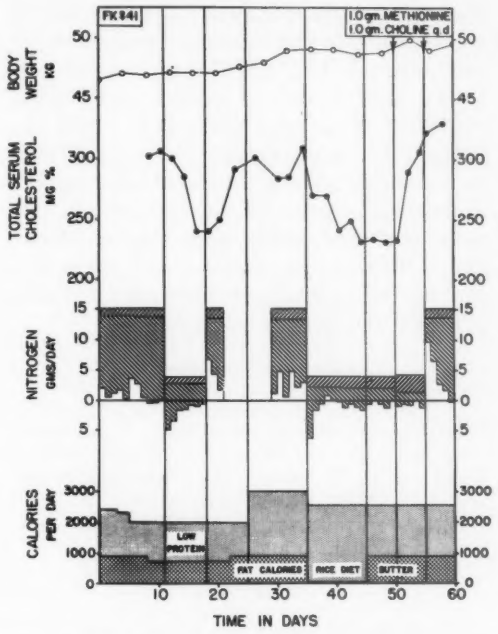


Fig. 4. Metabolic study of FK, female, 41 years. Body weight and serum cholesterol are plotted at the top. Next below, the dietary nitrogen is plotted upward from the zero line. Fecal nitrogen is then plotted downward from the intake line and urinary nitrogen is plotted cumulatively downward from that. The nitrogen balance is read directly as a displacement of the inverted bar from the zero line—positive above the zero line and negative below the zero line. Caloric intake is plotted at the base of the chart with calories contributed by fat at the bottom. Lipotropic therapy was given as indicated from the 50th to the 55th day.

SUMMARY AND CONCLUSIONS

Choline deficiency in the young adult rat causes a marked alteration in fat transport with an increase in liver fat and a decrease in the level of serum cholesterol, phospholipid, and triglyceride. There is a concomitant reduction in the concentration of the high-density α-lipoproteins and virtual disappearance of the low-density β-lipoproteins. Although added methionine (as casein) tended to prevent the hypolipemia in the rat, and ethionine causes a similar hypolipemia in the dog,[55] the effectiveness of choline in preventing the effect on low-methionine diets suggests that choline is the metabolite principally involved. Its precise mode of action in increasing mobilization of liver fat is still not known. In contrast to man, the degree of unsaturation of the dietary fat fed to the rat in the presence or absence of choline does not appear to affect its serum lipid pattern.

Studies in the human have shown that reduction of dietary protein and choline without altering dietary fat or calories causes hypocholesterolemia and hypo-β-lipoproteinemia in man. The amino acids possibly involved in this effect include methionine (which appears to be limiting), lysine, tryptophan, and threonine (which are marginal). The hypocholesterolemia does not parallel the degree of negative nitrogen balance and persists as long as the low-protein ration is fed. The failure of Keys and Anderson[56] to observe an effect of dietary protein upon serum cholesterol in schizophrenic men was probably due to the high range of dietary protein studied (64–138 g/day).

These studies support the central role of the liver in fat transport and show that dietary protein and choline may influence not only liver fat content but also the concentration of the serum lipids and lipoproteins in rat, dog, monkey, and man. It has been shown, further,

that the hypolipemia of kwashiorkor can be quickly corrected by the administration of fat-free milk powder.[57,58] Whether diets low in animal protein tend to protect adult human populations from atherosclerosis by virtue of a "defect" in fat transport is not clear. All of the available clinical and epidemiologic evidence would suggest that dietary protein, particularly animal protein, as well as fat, plays a role in the control of serum lipid levels in man and may be a significant environmental factor in the development of atherosclerosis.

ACKNOWLEDGMENTS

The authors should like to acknowledge the invaluable technical assistance of Senophia Gary, Walter Leskowitz, Janice Neville, Margaret Rainwater, Edmund Taylor, and Carole Yakin. The nutrient supplements used in this study were donated by the J. B. Roerig Company (Viterra®) and the U. S. Vitamin Corporation (Methiscol®), both of New York, New York.

REFERENCES

1. Dawber, T. R., Moore, F. E., and Mann, G. V.: Coronary heart disease in the Framingham Study. *Am. J. Pub. Health* 47: 45, 1957.
2. Olson, R. E., Vester, J. W., Gursey, D., and Longman, D.: The effect of low protein diets upon serum cholesterol in man. *J. Clin. Investigation (abstr.)* 36: 917, 1957.
3. Olson, R. E.: The role of dietary fat in human nutrition. V. Summary. *Am. J. Pub. Health* 47: 1538, 1957.
4. Anitschow, N.: Experimental atherosclerosis; in Cowdry, E. V.: *Arteriosclerosis: A Survey of the Problem.* Macmillan, New York, 1933.
5. Gofman, J. W., Jones, H. B., Lindgren, F. T., Lyon, T. P., Elliott, H. A., and Strisower, B.: Blood lipids and human atherosclerosis. *Circulation* 2: 161, 1950.
6. Rokitansky, C.: *Handbuch der Pathologische Anatomie* (1846), cited by Long in Cowdry, E. V.: *Arteriosclerosis, A Survey of the Problem.* Macmillan, New York, 1933.
7. Duguid, J. B.: Diet and coronary disease. *Lancet* 1: 891, 1954.
8. Lansing, A. I., Alex, M., and Rosenthal, T. B.: Atheromatosis as a sequel to senescent changes in the arterial wall. *J. Gerontol.* 5: 314, 1950.
9. Moon, H. D. and Rinehart, J. F.: Histogenesis of coronary arteriosclerosis. *Circulation* 6: 481, 1952.
10. Virchow, R.: Gesammelte abhandlunger zur wissenschaftlichen. Medicine Frankfurt Meidinger Sohn u Comp., 1856.
11. Page, I. H.: Atherosclerosis: An introduction. *Circulation* 10: 1, 1954.
12. Lewis, J. H. and Didischeim, P.: Differential diagnosis and treatment in hemorrhagic disease. *Arch. Int. Med.* 100: 157, 1957.
13. Bloom, B., Chaikoff, I. L., Reinhardt, W. O., Entenman, C., and Dauben, W. G.: The quantitative significance of the lymphatic pathway in the transport of absorbed fatty acids. *J. Biol. Chem.* 184: 1, 1950.
14. Chaikoff, I. L., Bloom, B., Siperstein, M. D., Kiyasu, J. Y., Reinhardt, W. O., Dauben, W. G., and Gastham, J. F.: C^{14}-cholesterol I. Lymphatic transport of absorbed cholesterol-4-C^{14}. *J. Biol. Chem.* 194: 407, 1952.
15. Friedman, M., Byers, S. O., and Rosenman, R. H.: Observations concerning the production and excretion of cholesterol in mammals; XIX. Demonstration of the essential role of the hepatic reticulo-endothelial cell (Kupffer cell) in the normal disposition of exogenously derived cholesterol. *Am. J. Physiol.* 177: 77, 1954.
16. Hellman, L., Rosenfeld, R. S., Eidinoff, M. L., Fukushien, D. K., Gallagher, T. F., Wang, C. I., and Adlersberg, D.: Isotopic studies of plasma cholesterol of endogenous and exogenous origins. *J. Clin. Investigation* 14: 48, 1955.
17. Friedman, M., Byers, S. O., and Michaelis, F.: Production and excretion of cholesterol in mammals. *Am. J. Physiol.* 164: 789, 1951.
18. Entenman, C., Chaikoff, I. L., and Zilversmit, D. B.: Removal of plasma phospholipids as a function of the liver: the effect of exclusion of the liver on the turnover rate of plasma phospholipids as measured with radioactive phosphorus. *J. Biol. Chem.* 166: 15, 1946.
19. Mann, F. C.: Modified physiologic processes following total removal of the liver. *J.A.M.A.* 85: 1472, 1925.
20. Lipsky, S. R., McGuire, J. S., Jr., Bondy, P. K., and Man, E. B.: The rates of synthesis and transport of plasma fatty acid fractions in man. *J. Clin. Investigation* 34: 1760, 1955.
21. Bates, M. W.: Turnover rates of plasma lipids. *Fed. Proc.* 17: 186, 1958.
22. Korn, E. D. and Quigley, T. W.: Studies on lipoprotein of rat heart and adipose tissue. *Biochim. and Biophys. Acta* 18: 143, 1955.
23. Dole, V. P.: Transport of non-esterified fatty acids in plasma; in *Chemistry of Lipide as Related to Atherosclerosis* (ed. by I. H. Page). Thomas, Springfield, Ill., 1958, pp. 189, 197.
24. Albrink, M. J. and Man, E.: Serum triglycerides in relation to carbohydrate metabolism in health and diabetes. *Diabetes* (in press).
25. Seifter, J. and Baeder, D. H.: Role of the liver on consequences of lipid mobilization. *Proc. Soc. Exper. Biol. & Med.* 95: 747, 1957.
26. Pomeranze, J., Beinfield, W. H., and Chessin, M.: Serum lipid and fat transport studies in

normal, obese, and atherosclerotic subjects. *Circulation* 10: 742, 1954.

27. LABECKI, T.: Hyperchylomicronemia and hypercholesterolemia: Their correlation with clinical atherosclerosis. AM. J. CLIN. NUTRITION 3: 132, 1955.
28. KEYS, A.: Atherosclerosis: A problem in newer public health. *J. Mt. Sinai Hosp.* 20: 118, 1953.
29. KEYS, A.: Diet and the epidemiology of coronary heart disease. *J.A.M.A.* 164: 1912, 1957.
30. YERUSHALMY, J. and HILLEBOE, H. E.: Fat in the diet and mortality from heart disease; a methodologic note. *New York J. Med.* 57: 2343, 1957.
31. YUDKIN, J.: Diet and coronary thrombosis; hypothesis and fact. *Lancet* 2: 155, 1957.
32. OLSON, R. E., JABLONSKI, J. R., and TAYLOR, E.: Effect of dietary protein, fat and choline upon the serum lipids and lipoprotein of the rat. AM. J. CLIN. NUTRITION 6: 111, 1958.
33. MORRISON, L. M.: A nutritional program for prolongation of life in coronary atherosclerosis. *J.A.M.A.* 159: 1425, 1955.
34. GRIFFITH, W. H. and WADE, N. J.: Choline metabolism. I. The occurrence and prevention of hemorrhagic renal degeneration in young rats on a low choline diet. *J. Biol. Chem.* 131: 567, 1939.
35. DE LALLA, O. F. and GOFMAN, J. W.: Ultracentrifugal analysis of serum lipoproteins; in *Methods of Biochemical Analysis*. Interscience Publishers, New York, 1954, p. 459.
36. BOYD, E. M.: Species variation in normal plasma lipids estimated by oxidative methods. *J. Biol. Chem.* 143: 131, 1942.
37. GREEN, A. A. and PAGE, I. N.: Ultracentrifugal lipoprotein patterns of serum of normal, hypertensive and hypothyroid animals. *Am. J. Physiol.* 171: 391, 1952.
38. WILGRAM, C. F., LEWIS, L. A., and BLUMENSTEIN, J.: Lipoproteins in protein deficiency. *Circulation Res.* 3: 549, 1955.
39. OLSON, R. E.: Effect of dietary protein upon fat transport. *Diabetes* (in press).
40. RIDOUT, J. H., LUCAS, C. C., PATTERSON, J. M., and BEST, C. H.: Effects of lipotropic substances on the cholesterol content of the serum of rats. *Biochem. J.* 58: 306, 1954.
41. WILGRAM, G. F., LEWIS, L. A., and BEST, C. H.: The effect of choline and cholesterol on lipoprotein patterns of rats. *Circulation Res.* 5: 111, 1957.
42. STETTEN, DEW. and SALCEDO, J.: The source of extra liver fat in various types of fatty liver. *J. Biol. Chem.* 156: 27, 1944.
43. MCKIBBON, J. M., THAYER, S., and STARE, F. J.: Choline deficiency studies in dogs. *J. Lab. & Clin. Med.* 29: 1109, 1944.
44. ZILVERSMIT, D. B., ENTENMAN, C., and CHAIKOFF, I. L.: The measurement of turnover of the various phospholipides in liver and plasma of the dog and its application to the mechanism of action of choline. *J. Biol. Chem.* 176: 193, 1948.
45. MANN, G. V., ANDRUS, S. B., MCNALLY, A., and STARE, F. J.: Experimental atherosclerosis in Cebus monkeys. *J. Exper. Med.* 98: 195, 1953.
46. KEMPNER, W.: Treatment of hypertensive vascular disease with the rice diet. *Am. J. Med.* 4: 545, 1948.
47. KEYS, A., MICKELSEN, O., MILLER, E. V. O., and CHAPMAN, C. B.: The relation in man between cholesterol levels in the diet and in the blood. *Science* 112: 79, 1950.
48. STARKE, H.: Effect of rice diet on the serum cholesterol fractions of 154 patients with hypertensive vascular disease. *Am. J. Med.* 9: 494, 1950.
49. WATKIN, D. M., FROEB, H. F., HATCH, F. T., and GUTMAN, A.: Effect of diet in essential hypertension. II. Results with unmodified Kempner rice diet in fifty hospitalized patients. *Am. J. Med.* 9: 441, 1950.
50. CHAPMAN, C. B., GIBBONS, T., and HENSCHEL, A.: The effect of the rice-fruit diet on the composition of the body. *New England J. Med.* 243: 899, 1950.
51. KEYS, A., ANDERSON, J. T., and GRANDE, F.: Serum cholesterol response to dietary fat. *Lancet* 1: 787, 1957.
52. ORR, M. L. and WATT, B. K.: The amino acid content of foods. Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C., 1955.
53. ROSE, W. C., WIXOM, R. L., LOCKHART, H. B., and LAMBERT, G. F.: The amino acid requirements of man. XV. The valine requirement; summary and final observations. *J. Biol. Chem* 217: 987, 1955.
54. KARK, R. M., HORWITT, M. K., and ROTHWELL, W. S.: Production and repair of liver dysfunction in man by modifications of dietary protein. *J. Lab. & Clin. Med.* 42: 823, 1953.
55. FEINBERG, H., HILL, R., RUBIN, L., and CHAIKOFF, I. L.: Reduction of serum lipids and lipoproteins by ethionine feeding in the dog. *Science* 120: 317, 1954.
56. KEYS, A. and ANDERSON, F. T.: Dietary protein and the serum cholesterol in man. AM. J. CLIN. NUTRITION 5: 29, 1957.
57. SCRIMSHAW, N. S., BÉHAR, M., ARROYAVE, G., VITERI, F., and TEJADA, C.: Characteristics of kwashiorkor (syndrome pluricarencial de la infancia). *Fed. Proc.* 15: 877, 1956.
58. FRENK, S., GÓMEZ, F., RAMOS-GALVÁN, R., and CRAVIOTO, J.: Fatty liver in children—kwashiorkor. AM. J. CLIN. NUTRITION 6: 298, 1958.

DISCUSSION

Dr. M. K. Horwitt (University of Illinois, College of Medicine, Urbana, Ill.): The part of this which interests me the most and I think deserves a little more comment than it has had in the past two days, is an evaluation of the cholesterol level in the blood as a function of changes in metabolism. In other words, we have not said enough about the most important source of cholesterol, the endogenous source. The fact is that the liver has to be healthy in order to make cholesterol, and any liver which is inadequate, for nutritional or other reasons, possibly will not produce cholesterol at a rate which is considered normal.

Important among the factors which might affect the levels of cholesterol in the blood, or might affect cholesterol synthesis, are the toxic compounds. I think it is pretty well known that chloroform, phosgene, and vanadium will give you a type of liver dysfunction which will lower cholesterol in the blood as a secondary effect to the production of fatty livers. Starvation will lower cholesterol, as will exercise. There is no better demonstration of the lowering cholesterol than one I saw within the past two weeks, when we gave a good dose of triiodo-thyronine to a group of individuals and cholesterol in serum dropped from an average of 180 to an average of about 130 mg/100 ml in a ten-day period.

If these cholesterol levels are indeed lowered by liver pathology or liver dysfunction, we may have to think of a protein depleted diet as being one which supplies insufficient amino acids to make the proper enzyme systems to synthesize cholesterol. In the absence of protein one does not have optimum liver function, nor does one obtain the normal levels of cholesterol.

This of course has been demonstrated indirectly in a study at Elgin of patients after a five-month period on a diet producing approximately 45 g of protein. Dr. Kark and the other clinicians working with him found what they thought to be incontrovertible evidence of liver dysfunction both by physical diagnosis and by interpretation of bromsulphalein retention tests. The levels of the bromsulphalein which were noted in our patients after five months on the low-protein diet ranged as high as 18 per cent retention. After a regimen of increased protein, the bromsulphalein retention tests gave more normal data. Because of the lateness of the hour, I decided not to use any slides to demonstrate this and perhaps to leave some time for Dr. Kark to make some comments about those particular experiments.

The major point in this discussion is to call attention to the fact that many of the procedures that we might use to lower cholesterol in the blood might possibly be doing this by decreasing other physiologic functions. I don't want to imply that when we treat patients with unsaturated fatty acids the unsaturated fatty acids decrease the ability of the liver to synthesize cholesterol, but to date I have not found any proof that they do not.

Dr. M. Kark: I have nothing more to add.

Dr. Artom: I do not know if this is the place to discuss experiments on rats, since Dr. Olson has been talking mostly about lipids in human serum. But I have been very much interested in his experiments on rats because Dr. Lofland and I have done similar experiments with rats on a somewhat different diet, containing 8 per cent casein. At first with this diet we had about the same results that Dr. Olson had with his 18 per cent soy protein diet; that is, very low levels of all serum lipids, which are raised considerably by supplementing the diet with choline. This seemed to be in line with the idea that choline favors the mobilization of lipids from the livers.

However, the results were different when we modified the diet. I hesitate to report these results, because thus far we have not made many experiments. But when, for instance, we tried to make our diet a little better by adding some threonine and some cystine, we were unable to detect any significant effect of choline when this was also added to the diet.

In no case have we seen a sudden increase in the serum lipids after choline was given by injection let us say one hour, or two hours, before the blood was drawn. So it seems that when there is an effect of choline on serum lipids, this is apparent only after the substance has been given for a prolonged period. This would perhaps tie these results with the synthesis of the protein moiety of lipoproteins rather than with a mobilization of the free lipids.

Dr. Gabuzda: I might ask one brief question of Dr. Wilgram. Does he think that Dr. Olson's diet would produce fatty liver lesions in his monkeys?

Dr. Wilgram: Dr. Olson, may I recapitulate: How high was the protein content of your diet?

Dr. Olson: Each subject received 25 g a day. Twenty-five out of about 500 g of total solids would give about 5 per cent of total protein.

Dr. Wilgram: This was first-class protein, was it?

Dr. Olson: No, it was not first-class protein. It was vegetable protein, legumes, cereals, and certain other vegetables.

Dr. Wilgram: I am sorry, Dr. Gabuzda, I cannot answer this offhand. I apologize for this. The diets we use at the moment contain 10 per cent soya protein and 10 per cent alcohol-extracted peanut meal. While this gives 20 per cent of protein as such by weight, it actually gives only 12 per cent good protein. I rather feel that the same diet used by Dr. Olson would produce some degree of fatty liver in our animals. However, this is just an opinion.

Dr. Zilversmit: I want to make one comment on Dr. Olson's statement that in his hypolipotropic diets the blood serum lipids go down and at least in private conversations he has implied to me that on hypolipotropic diets you are bound to get low serum lipids. I think Dr. Wilgram also made such a statement.

This is true I feel in part. Certainly with choline you seem to get higher lipid levels than without choline in the diet. On the other hand, the type of hypolipotropic diet which we use and which was described yesterday will give much higher serum lipid levels (cholesterol, phospholipids, and triglycerides) than rabbit chow.

So this hypolipemia is a relative affair. In absolute terms you can obtain definite hyperlipemias in certain animals fed diets that are very low in lipotropic agents.

Dr. Jack D. Myers: Since there is interest in any alteration of other liver functions concomitant with this depression of cholesterol, the Chair might comment that some years ago we studied a group of patients on the Kempner rice diet, low in fat and low in protein, using the bromsulphalein clearance technic, which is a much more delicate technic than the standard BSP test for hepatic function (*Stanford Med. Bull.* 13: 2, 1955). In such patients, along with the reduction in serum in cholesterol, one can quite regularly demonstrate a retention of bromsulphalein. Whether these two phenomena are connected is still an open question. There certainly seems to be some liver dysfunction when one tinkers with the diet in the way we have heard described.

Dr. Olson (closing remarks): With regard to Dr. Horwitt's mention of a possible effect of low-protein feeding upon the endogenous synthesis of cholesterol in the liver, the point is very well taken. We really do not understand as yet the mechanism by which low-protein or hypolipotropic diets reduce the serum lipids in both rat and man, nor are we necessarily saying that lowering the serum lipids in this manner is "good." Our preliminary isotopic experiments employing acetate-1-C^{14} as a tag for newly synthesized cholesterol in the rat, indeed suggest that there may be some decrease in the biosynthesis of hepatic cholesterol in the choline-deficient rat. Whether this is also true in man we cannot say, although we are hoping to study this in the near future. We know that there is a considerable flexibility in the synthesis of cholesterol by the liver, depending upon the intake of cholesterol, but the extent that this is influenced by other nutrients has not yet been determined.

With regard to the comparison between our studies and those of Drs. Kark and Horwitt (*J. Lab. & Clin. Med.* 42: 823, 1953), I should like to ask either Dr. Kark or Dr. Horwitt which amino acid was limiting in their studies. As I recall, the object of these experiments was to explore the tryptophan-niacin interrelationship.

Dr. Horwitt: Threonine, lysine, and tryptophan were all borderline.

Dr. Olson: In our diets, which supply only 4.0 g of nitrogen as compared with the 6.5 g fed in yours, the limiting amino acid appears to be methionine although lysine, threonine, and tryptophan are present in marginal amounts. As already mentioned, our patients are in negative nitrogen balance for as long as ten weeks, but the extent of the negative balance during the last three weeks is as low as 0.1–0.2 g daily. Further, the extent of the negative nitrogen balance does not appear to bear a direct relationship to the extent of the hypocholesterolemia. It also would seem, in reference to Dr. Myer's comment about bromsulphalein retention in patients fed the rice diet, and to the changes observed by Dr. Kark and his group that this evidence of liver dysfunction comes much later than the hypocholesterolemia which is prompt and reaches a basement level in two to three weeks. In our patients, studied thus far for shorter periods, no bromsulphalein retention has been noted. On the other hand, most observers agree that severe parenchymatous liver disease resulting in crippling cirrhosis or hepatic coma, for example, results in a drop in total as well as ester serum cholesterol.

As regards the mechanism of the "corn oil effect" upon serum lipids, Hellman, Rosenfeld, Insull, and Ahrens (*J. Clin. Investigation* 36: 898, 1957) reported to the American Society of Clinical Investigation last spring that an isotope study of a hypercholesterolemic subject given butter and then corn oil as the sole source of dietary fat revealed that the feeding of corn oil was associated with a greater excretion of radioactive sterol in the stool without a change in the slope of the specific activity-time plot of serum cholesterol. Whether the effect is primarily one of biliary excretion or intestinal absorption is not yet determined.

Dr. Artom's comments in reference to his own observations on the effect of an 8 per cent casein diet in causing general hypolipemia in the rat is of great interest to us. I am not surprised to learn that the administration of choline to his animals on this regimen did not cause an immediate effect upon serum lipids. In our patients, the return of the serum cholesterol to normal or hypernormal with change from a 25 to a 100 g protein diet takes several days. In the patient fed the low-protein diet for ten weeks and then returned to the control 100 g protein diet, the serum cholesterol went from 213 to 317 mg per 100 ml, a hypernormal value, over the course of two weeks.

With regard to the suggestion that specific amino acids may control the elaboration of the protein moiety of the β-lipoproteins, and hence account for the "low-protein" effect, this is a definite possibility and has not been ruled out by any data which we have at the moment.

As to Dr. Gabuzda's question to Dr. Wilgram, regarding the comparability of our experiments in man and Dr. Wilgram's in the monkey, we do not know any factor which will provide exact transposition of data from one species to another. As regards the negative liver biopsies which we have obtained in our patients on low-protein diets, we have guessed that on the basis of comparative metabolic rates, or comparative life spans, a diet which produces a marked fatty change in a rat liver in one week, and our 25-g protein diet does, should produce some change in a human liver in 10

weeks. Mice respond to given hypolipotropic diets with much less fatty infiltration than do rats, whereas on a "metabolic rate yardstick" they should be worse. We feel that there are definite species variations in the response to hypolipotropic nutrition, some being explained by variations in C_1-metabolism and some as yet unexplained.

Finally, with reference to Dr. Zilversmit's comment, I would say that there is no doubt that various absolute concentrations of serum lipid can be obtained in studies of choline deficiency in different species, particularly, when the fat content of the diet is varied. The rat is not sensitive to the fat intake, as regards serum lipids, whereas the dog is moderately sensitive, and the rabbit maximally so, probably because of a basic species-linked clearing defect. We can only say that in choline deficiency in different species there is relative hypolipemia, and in some instances it is absolute.

Effect of Lipotropic Factors Upon Serum Lipids and Vascular Disease in Man

THADDEUS D. LABECKI, M.D., F.A.C.P.*

IT HAS BECOME almost universally accepted that disturbance in lipoprotein metabolism is but one phase in the chain of events leading to atheroma in man. It is obvious that since we have little or no control over other contributing factors,[1] our efforts must be aimed at the correction of the disturbed lipoprotein metabolism, through dietary or other means.

The purpose of this presentation is to summarize the position which the so-called lipotropic factors occupy in the admittedly poor armamentarium with which we have to combat vascular disease in man. They have been administered for the last several years not as palliative agents (which vascular dilating agents are) but as allegedly therapeutic substances aimed to correct at least one abnormality leading to the causation of atheroma. A few investigators have also expressed the hope that regression of the lesions might be induced but so far that remains in the realm of animal experimentation. It is perhaps fitting that at the close of this Symposium a bit of soul searching be indulged in; and, instead of reviewing further the subjects already discussed by other speakers, I will limit myself to posing certain clinical questions and attempt to answer them, if at all possible.

The questions to be discussed are the following: (1) What, if any, is the effect of lipotropic substances on the serum lipids in human beings? (2) Is there reasonable justification to administer those substances as preventive and/or therapeutic agents? (3) Should we properly include other substances (other than choline, methionine and inositol) into that traditionally esoteric "family" of lipotropic agents? (4) If so, what is the effect, if any, of those "other lipotropic substances?"

An aberration in lipid metabolism can reflect itself in a number of ways, and various indices have been employed to evaluate lipid and lipo protein metabolism of man. Historically and because of expediency, total—and at times esterified—serum cholesterol have been most frequently studied both in humans and in experimental animals. It will be out of place to contrast the group correlation with the significance in an individual case. There is an agreement among most, if not all of us, that as a group the premature and/or far-advanced coronary atherosclerosis subjects show higher serum cholesterol levels than do individuals with no, much less pronounced, or late atherosclerosis.[2–4] Similarly, in studies of populations allegedly suffering less from coronary artery disease, lower total serum cholesterol levels have been demonstrated than in comparable Western populations. Since the effect of lipotropic factors on total serum cholesterol levels, particularly in human beings, has been a matter of considerable controversy (*Am. J. Med.* 11:107, 1951) I present herein the results of a previously published study of ours in which serum cholesterol and chylomicron counts were carried out in a group of patients with myocardial infarction and in a group of "healthy" controls.[2] The so-called infarction group (Table I) consisted of 77 patients, all of whom presented unequivocal electrocardiographic evidence of myocardial infarction in the past. There were 216 subjects in the non-infarction group; of these 139 were 55 years of age or

* Medical Consultant, Heart Disease Control Unit, Mississippi State Board of Health, Jackson, Mississippi.

Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc., New York, N. Y.

TABLE I

Composition of Groups Under Observation[3]

Age group	Non-infarction group					Infarction group				
	Total	WM	WF	CM	CF	Total	WM	WF	CM	CF
Under 45 years	28	8	20	0	0	14	13	0	1	0
45–54 years	49	19	7	9	14	20	17	1	1	1
55–64 years	42	7	9	17	9	29	22	5	1	1
65–74 years	34	16	14	2	2	11	8	2	1	0
75 years and over	63	13	50	0	0	0	0	0	0	0
Not stated	0	0	0	0	0	3	2	0	1	0
Total	216	63	100	28	25	77	62	8	5	2

WM—white male; CM—colored male: WF—white female; CF—colored female.
From: Labecki, T. D.: Am. J. Clin. Nutrition 3: 132, 1955.

older. The difference between the means for the total cholesterol of the two groups was four times higher than the standard error of difference.

The chylomicron levels referred to in Table II mean the percentage of particles 0.3 μ in diameter and larger in relation to the total number of visible fatty particles as studied by the technic described by us.[2] The difference between the means of the infarction and non-infarction groups, as far as chylomicron levels are concerned, was 9.2 times the standard error. When subjects were divided into groups according to age, the patients with infarction showed surprisingly similar mean chylomicron ratios in all age groups and the ratios were always statistically significantly higher than the mean group values for the non-infarction subjects.

Sixteen patients with electrocardiographically proved myocardial infarction in the past were put on lipotropic regime, consisting of 2 g of choline, 1 g of methionine, over 600 mg of inositol administered in capsule form. Because we have observed a significant increase of the chylomicron levels in the presumably non-atherosclerotic females 65 years of age and older,[2] it was deemed advisable to administer the lipotropic substances to 26 white females with a mean age of 77 years, all of whom are

TABLE II

Mean Value, Standard Deviation, and Standard Error of Mean of Chylomicron Level and Total and Esterified Cholesterol[3]

Descriptive measurements	Non-infarction group*	Infarction group
Chylomicron level		
Persons tested	136	75
Mean, %	35.8	42.7
Standard deviation	7.65	7.1
Standard error of mean	0.65	0.82
Cholesterol		
Persons tested	137	68
Mean, mg per 100 ml	266.7	304.9
Standard deviation	56.7	67.9
Standard error of mean	4.9	8.2
Cholesterol esters		
Persons tested	128	66
Mean, mg per 100 ml	188.4	200.8
Standard deviation	43.2	50.7
Standard error of mean	3.8	6.2

* Excludes all persons under 55 years of age.
From: Labecki, T. D.: Am. J. Clin. Nutrition 3: 135, 1955.

TABLE III

Effect of Administration of Lipotropic Substances on Fasting Chylomicron Levels[5]

Period of administration, weeks	4	9	12
Non-infarction group			
Number of persons	25	24	11
Mean difference	−5.56	−10.36	−8.19
S.D.	4.73	6.34	3.22
Statistical significance	Yes	Yes	Yes

Period of administration, weeks	Up to 9	9 or more
Infarction group		
Number of persons	11	11
Mean difference	−7.94	−15.72
S.D.	6.16	7.57
Statistical significance	Yes	Yes

− Signifies decrease (see reference [5] for details).

TABLE IV

Effect of Administration of Lipotropic Substances on Total Serum Cholesterol and Cholesterol Esters[5]

Period of administration, weeks	4	9	12
Non-infarction group			
Cholesterol			
Number of persons	25	24	12
Mean difference*	+11.16	−0.02	+0.29
S.D.	20.80	20.42	26.50
Statistical significance	Borderline	No	No
Cholesterol esters			
Number of persons	25	24	12
Mean difference*	−6.22	+0.79	+4.38
S.D.	20.16	25.27	43.23
Statistical significance	No	No	No

Period of administration, weeks	Up to 9	9 or more
Infarction group		
Cholesterol		
Number of persons	12	11
Mean difference*	−13.46	−25.44
S.D.	30.42	37.50
Statistical significance	No	Borderline
Cholesterol esters		
Number of persons	12	11
Mean Difference*	+2.25	−14.80
S.D.	28.37	46.50
Statistical significance	No	No

− Signifies decrease; + signifies increase (see reference [5] for details).

* Mean difference in mg/100 ml.

permanent residents of a home for aged ladies.

Table III shows the effect of administration of lipotropic substances on fasting chylomicron levels. In both the infarction and non-infarction groups there was a statistically significant depression of chylomicron levels following rather intensive therapy with lipotropic agents. This effect seems to become demonstrable three to four weeks after initiation of an adequate regime and continues during the period of administration and for a short time following discontinuation. This "after effect" varies in duration from individual to individual but usually lasts for several weeks.[5]

Table IV shows the effect of administration of lipotropic substances on total serum cholesterol and cholesterol esters in the non-infarction group and in the infarction group. In the group with myocardial infarction prolonged administration of the lipotropic factors—that is, nine weeks and longer—resulted in borderline statistical significance in the lowering of total serum cholesterol levels. There was little effect on the cholesterol levels if the regime was continued for a period of less than nine weeks. During the first four weeks of administration of the lipotropic substances to the non-infarction group, there was an increase in the total cholesterol levels which was of borderline statistical significance.

Since the effect on chylomicron levels appeared to be of considerable statistical significance, the effect should be viewed against the known mean variability in the chylomicron levels. This mean variability for 31 individuals tested on repeated occasions during one year was 2.3 per cent. For 16 individuals repeatedly tested by the same technical personnel during the second year of the study, it was 1.7 per cent, giving an over-all mean for the two-year period of 2.0 per cent. The split samples of the same tests performed blindly rarely differed by more than 2.0 per cent. In our hands a comparable mean value for cholesterol variation was 17.9 mg/100 ml and 12.9 mg/100 ml for the esterified fraction of cholesterol.[5]

Our findings summarized above were subsequently confirmed by Rawls and associates and recently published.[6]

The effect of lipotropic agents in lowering the chylomicron levels appears to be surprisingly consistent. Whether the action of choline and methyl-group donors like methionine and betaine and whether the action of inositol is to provide components of phospholipids and thus increase the phospholipid serum content should be studied further. Such an increase might be reflected, from the physical standpoint, by the increase in the smaller (0.1 to 0.2 micron) visible fatty particles and perhaps other particles in the ultra-microscopic segment, thereby causing a relative decrease in the chylomicron concentration. Thus the action of lipotropic substances may be postulated as creating a shift in the levels of lipoprotein macromolecules of various sizes. This might very well be the ex-

planation of the cholesterol-stabilizing effect of the phospholipids.

Among the indices of atherogenesis, as far as lipoprotein metabolism is concerned, the relative concentration of α- and β-lipoproteins expressed conveniently as α-to-β-lipoprotein ratio has become, in the opinion of many, the most reliable and sensitive index. Again it would be superfluous to cite the data of other investigators.

In our study[7] 21 individuals were chosen as control subjects, 19 males and 2 females, ranging in age from 10 to 44 years and including one 3-year-old child.* The basic requirement was the absence of clinically demonstrable disease of cardiovascular or any other nature. A second "control" consisted of pooled serum from healthy blood donors. As the experimental group, 25 patients, 16 males and 9 females, age 36 to 78 years, with unequivocal evidence of coronary atherosclerosis (as evidenced by one or more attacks of myocardial infarction)—and who have been under our continuous observation for from four to five years with monthly determination of serum lipids—were chosen as subjects for this study.

The range of α- to β-lipoprotein ratio for the control group varied from 0.41 to 1.10 with the mean of 0.729. The means on multiple runs of pooled serum from presumably healthy blood donors varied from 0.62 to 0.67. It was gratifying that the relatively small group of our control individuals differed insignificantly from the mean of the pooled serum which represented sera of many clinically healthy individuals.

The α- to β-lipoprotein ratio for the coronary group, however, was 0.461. The mean α-to-β-lipoprotein ratio in the coronary group differs statistically significantly from the mean for the control group, i.e. both the group of 21 individuals and pooled serum samples.

Because of our experience with the apparent synergistic action of lipotropic agents, we administered those substances to a group of patients with coronary artery disease and studied the α- and β-lipoproteins and cholesterol levels at monthly intervals. We could detect no shift in α- to β-lipoproteins; indeed we were impressed by the reproducibility of the results from test to test and by the constancy of the lipoprotein levels either with or without lipotropic regime.

Sinclair, and others[8,9] have postulated that atherosclerosis is possibly intensified by a dietary deficiency of the unsaturated fatty acids such as linoleic, and arachidonic. Our next step was stimulated by this and other reports[10] on the effect of certain vegetable oils rich in essential unsaturated fatty acids on serum lipids. Because I question the advisability of administering large doses of fatty acids, either saturated or unsaturated to individuals with presumably disturbed lipoprotein metabolism, I decided to administer rather moderate doses, in capsule form, of safflower oil which is a rich source of linoleic acid, an acid which, as is well known, possesses a very high iodine number. Administering doses of 4 g of safflower oil per day over a prolonged period of time produced *no* demonstrable shift in the α- and β-lipoproteins or in cholesterol levels.

I would like to summarize our experience when we combined lipotropic substances, namely, 1 g of DL-methionine, 750 mg of inositol and 1 g of choline with 3 g of safflower oil yielding approximately 2 g of linoleic acid daily. In capsule form this was supplemented with 4 mg daily of pyridoxine in divided doses. The latter inclusion was due to the several reports, notably that of Whitten and Holman[11] which have shown that pyridoxine is necessary for the formation of arachidonic and hexaenoic acid from linoleic and linolenic acids. This suggests that the interrelation between pyridoxine and those acids is manifested by a sparing effect of one when deficiency of the other is induced. The results which will be briefly summarized here are the subject of a separate publication.[7] It is gratifying that the trend which we detected up to a 30-week interval has been maintained and we have that confirmation by subsequent monthly checks.

The action of safflower oil combined with lipotropic substances effected an increase ratio of α-to-β-lipoproteins which was in evidence at a 12-week interval, but when submitted to statistical analysis was found to be of borderline statistical significance for that duration of

* The α- and β-lipoproteins were determined by our modification of paper electrophoresis.[7]

TABLE V

Effect of the Administration of Combined Safflower Oil and Lipotropic Therapy upon Serum Lipoproteins

Serum lipoproteins	Control group	Infarction group		
		0	18 Wks. of therapy	Difference observed
No. persons	21	25	21	—
α:β-lipoprotein ratio	0.729	0.461	0.580	—
Mean difference				0.119
Standard deviation	0.194	—	0.170	0.02
Standard error	0.040	—	0.037	0.003

therapy. At the 18-week mark, findings in 21 out of 25 patients were as follows (four cases were omitted because samples were unavailable at the 18-week mark): The average α-to-β-lipoprotein ratio before administration of safflower oil plus lipotropic substances was 0.461 and changed to 0.580 after 18 weeks of therapy, as shown in Table V. The odds against occurrence of a deviation as great as, or greater than 0.12 (the increase in the α-to-β-lipoprotein ratio after 18 weeks) are approximately two million to one. When we compare the presumed normal group with the treated group of 21 patients after 18 weeks of therapy, we find a difference between the two means of 0.149, still in favor of the presumed normal group. Although a significant increase in the α-to-β-lipoprotein ratio was observed after administration of safflower oil combined with lipotropic substances, the average ratio for the 21 coronary patients under consideration is still statistically significantly lower than that for the presumed normal group. Six out of 25 "coronary" patients showed a definite decrease in total serum cholesterol levels at the 18- or 30-week period.

Our experimental approach differed from other studies in several respects; (1) Instead of replacing animal fats in the diet, we supplemented the normal diet by moderate doses of a substance rich in unsaturated fatty acids and combined it with lipotropic substances. (2) Although we also were determining total serum cholesterol, we emphasized the α-lipoprotein to β-lipoprotein ratio as a sensitive index; permanent records are maintained for future reference and as a double check of the efficacy of the regimen. (3) In view of the dislike of many patients to ingest large amounts of a drug and because of our firm conviction that it may not be conducive to their welfare to administer large doses of vegetable oil, we used moderate doses of a substance rich in the unsaturated fatty acid, linoleic acid. That our findings are not inconsistent with the results reported by others is proved by report of Kinsell and associates[12] who found that the intake of 2.0 g of linoleic acid over a 24-hour period resulted in a prompt and profound decrease in plasma cholesterol and other lipid fractions. Our moderate doses of safflower oil combined with lipotropic factors resulted in a decrease of cholesterol in 6 patients out of 25. In our laboratory the technical error for any one cholesterol determination is ± 14 per cent. Well-controlled daily cholesterol determinations, however, result in surprisingly little variation in serum cholesterol from month to month in a given patient as was demonstrated in the case of the subjects in this study.

We obtained no shift in the α-to-β-lipoprotein ratio and no change in the serum cholesterol levels when we administered lipotropic substances alone, or safflower-seed oil alone.

The meaning of the lipoprotein shift observed on combined therapy might be subjected to syllogistic reasoning; the α-to-β-lipoprotein ratio (high in young, healthy females) is markedly increased in patients under the influence of estrogenic substances or parenteral administration of heparin. The protective action of estrogenic substances and heparin are well known and disputed by few; the increase in the ratio should be considered as a salutary phenomenon from the patient's standpoint. I would like to make a sincere plea for others to follow this line of thinking and attempt to duplicate our results using similar technics and similar doses of the safflower seed-oil and lipotropic factors.

The question posed at the beginning of this report asked whether there is a justification for administering lipotropic factors as preventive and/or therapeutic agents, and the answer on the basis of objective studies, in my opinion, is positive. In light of our experience concerning

the apparent synergism between unsaturated fatty acids and lipotropic substances, the answer as to whether these nutritional factors should be combined, again is positive.

For a number of years, Dr. Louis Katz and his associates were strongly opposed to the concept of lipotropic factors playing any role in causation of atherosclerosis. I am happy to acknowledge that Dr. Katz and his associates changed their point of view.[13] In a recent paper, Katz said, "The key to understanding the essence of the atherosclerosis problem lies in appreciating that it is basically a metabolic disease. In particular, alterations in cholesterol-lipid-lipoprotein metabolism play a critical and decisive (but not exclusive) role in the pathogenesis of atherosclerosis. Without alterations in cholesterol-lipid-lipoprotein metabolism, clinically significant atherosclerosis would occur but rarely (if ever), particularly in middle age, regardless of the functional state of the cardiovascular system.

The contemporary American diet, a peculiar by-product of civilization and the socio-economics of our culture, is pernicious to the cardiovascular system not only because of its excess in calories, lipids, and cholesterol (estimates indicate that the average American business and professional man ingests almost 60% of his total calories in the form of fats) but also because it tends to be quite high in salt and in empty calories; i.e., calories derived from highly processed refined carbohydrates and fats (particularly animal fats and hydrogenated saturated vegetable fats), foods rich in energy but low in essential nutrients. Hence, the American diet, despite its high caloric level, is frequently inadequate, both relatively and absolutely, in specific vitamins, essential fatty acids, minerals, and amino acids. It is this acquired 'rich' and unbalanced diet that, by altering cholesterol-lipid-lipoprotein metabolism, creates the decisive prerequisites on a mass scale for the ready genesis of atherosclerosis in the American population."[13]

The administration of lipotropic factors and other substances such as unsaturated fatty acids is obviously a corrective therapy which has sound experimental background. Two decades ago it was considered pharmacologically inelegant to administer more than one vitamin at a time. A few years ago even the word vitamin implied a bit of quackery. I hope that we have also matured as far as realization of the importance of nutritional factors in metabolic processes is concerned and thus in the causation of atherosclerosis. Lipotropic factors and other nutrients obviously play considerably important roles in that complex problem.

REFERENCES

1. LABECKI, T. D.: The multi-factor concept of atherogenesis. Proc. Southern Reg. Meet. Am. College Phys., Jan. 1956.
2. LABECKI, T. D.: Hyperchylomicronemia and hypercholesterolemia: Their correlation with clinical atherosclerosis. AM. J. CLIN. NUTRITION 3: 132, 1955.
3. KATZ, L. N., STAMLER, J., and PICK, R.: Atherosclerosis and coronary heart disease. *Lancet* 76: 355, 1956.
4. PAGE, I. H.: Atherosclerosis; an introduction. *Circulation* 10: 1, 1954.
5. LABECKI, T. D., BRIGHT, I. B., LAKE, W. W., and THOMPSON, C. C.: Effect of a therapeutic regime on hyperchylomicronemia and hypercholesterolemia. AM. J. CLIN. NUTRITION 3: 141, 1955.
6. RAWLS, W. B. and TICHNER, J. B.: Lipid metabolism and the effect of a combined lipotropic agent. *J. Am. Geriatrics Soc.* 4: 89, 1956.
7. LABECKI, T. D., BRIGHT, I. B., LAKE, W. M., and THOMPSON, C.: α and β lipoproteins and serum cholesterol levels following administration of unsaturated fatty acids. *Proc. Soc. Exper. Biol. & Med.* 97: 260, 1958.
8. SINCLAIR, H. M.: Deficiency of essential fatty acids and atherosclerosis, et cetera. *Lancet* 1: 381, 1956.
9. BRONTE-STEWART, B., ANTONIS, A., EALES, L., and BROCK, L. F.: Effects of feeding different fats on serum-cholesterol level. *Lancet* 1: 521, 1956.
10. AHRENS, E. H., HIRSCH, J., INSULL, W., TSALTOS, T. T., BLOMSTRAND, R., and PETERSON, M. L.: The influence of dietary fats on serum-lipid levels in man. *Lancet* 1: 943, 1957.
11. WHITTEN, P. W. and HOLMAN, R. T.: Polyethenoid fatty acid metabolism. VI. Effect of pyridoxine on essential fatty acid conversions. *Arch. Biochem.* 41: 266, 1952.
12. KINSELL, L. W., FRISKEY, R. W., MICHAELS, G. D., and BROWN, F. R., JR.: Effect of a synthetic triglyceride on lipid metabolism. AM. J. CLIN. NUTRITION 4: 285, 1956.
13. KATZ, L. N., STAMLER, J., and PICK, R.: Research approach to atherosclerosis. *J.A.M.A.* 161: 536, 1956.

DISCUSSION

Dr. J. Vester (University of Pittsburgh, Pittsburgh, Pa.): As I understand Dr. Labecki's basic hypothesis, it is that the American diet, high in fat and protein, may provide a relative deficiency of lipotropic factors which in turn may influence lipoprotein metabolism in such a way as to be deleterious to health. Whether this hypothesis will stand the test of time and further scrutiny, however, is questionable in my mind because both the high-"fat" and the high-protein of the American diet supply fairly large amounts of lipotropic factors.

Nevertheless, I was very much interested in some of the observations which were presented. Neither the lipotropic supplement (which was ample) nor the safflower oil supplement (which was minimal) had an effect on serum cholesterol or α:β-lipoprotein ratio when given alone. This is not surprising in view of previous work with lipotropic supplements in man (Davidson, J. D., *Am. J. Med.* 11: 107, 1951) and of the studies of Ahrens *et al.* (*J.A.M.A.* 164: 1905, 1957) in which it was shown that the effect of corn oil feeding upon serum cholesterol was considerably reduced when this dietary fat was reduced from 40 per cent to 10 per cent of calories. Could the synergistic effect of this relatively low dose of safflower oil with lipotropic supplements be due to an alteration in the iodine number of the plasma fatty acids such as reported by Tobian and Tuna in patients fed supplemental corn oil? (*Clin. Res. Proc.* 5: 182, 1957)? Inasmuch as current estimates show that there is about 5 g of linoleic acid present in the current "rich, unbalanced" American diet (*Am. J. Pub. Health* 47: 1530, 1957) it seems somewhat improbable that an additional 2 g would appreciably influence the mean serum fatty acid unsaturation, but it remains a possibility. My last question relates to liver function. Is there any evidence that the therapy modified liver function and hence was instrumental in changing the α:β-lipoprotein ratio?

Dr. Kark: I wonder if Dr. Labecki could tell us the exact lipotropic content of the diet fed his control group and the group on therapy?

Dr. Olson: The Chair would like to ask one question: In terms of the change in ratio of α to β-lipoproteins, observed with safflower oil lipotropic therapy, does this represent an increase in α, a decrease in beta, or a combination of both? I presume your technic involves paper electrophoresis and oil-red staining of the strips with elution of cholesterol from the stained areas.

Dr. Labecki: I believe, Dr. Vester, it is quite correct that the dose of safflower oil is a critical factor in influencing serum cholesterol levels. I do question the advisability, as I mentioned before, of administering large doses of any vegetable oil, saturated or unsaturated. We try to reach a "compromise" level.

Your concept of change in the iodine number is a very intriguing possibility. That may be a possible explanation. I wholeheartedly agree with you that therapy in this field should aim to correct something which should have existed to begin with, either due to heredity or diet or other factors.

The lipotropic supplement to the diet, which was mentioned before, consisted of not less than 2 g of choline, 1.25 g of DL-methionine, close to 1 g of inositol, and a minimum of 3 g of safflower oil, yielding about 2 g of linoleic acid.

Dr. Kark: I don't think you understood my question. These people are eating a normal American diet, and I would like to know the inositol, choline, and methionine content of that diet.

Dr. Labecki: That is a very intriguing question. Unfortunately, they consumed a "normal" southern diet. I have failed so far to determine the lipotropic content. I suspect it is not very high because of the large amount of vegetables and relatively little meat and dairy products. It varies from individual to individual, but we make it a point not to change the dietary habits of the individual.

Dr Kark: The reason I bring up this point is that the normal northern diet in the U. S. A. contains large amounts of these lipotropic factors, and I can't see that adding extra lipotropic factors would tend to do anything to the northern population. It may certainly do so in your population.

Dr. Labecki: Possibly so. However, we may be dealing, as Dr. Vester mentioned, with the possibility of a relative deficiency; the more foods incorporating lipotropic substances consumed, the more calories consumed, thus creating a greater demand, for lipotropic substances. So there may be a relative deficiency even though we do increase the over-all intake of both calories and lipotropes.

Dr. Olson: May I interpose a question? What per cent of these patients have any liver dysfunction or evidence of fatty liver?

Dr. Labecki: We have not studied them as far as their liver function is concerned.

Dr. Olson: Will you answer my question about the relative changes in α- and β-lipoproteins?

Dr. Labecki: I am sorry. Yes, we use the method you mentioned.

Dr. Olson: Did the betas go down?

Dr. Labecki: We obtained a change in the α-β ratio with a decrease in β-lipoproteins.

Editorial

*Vitamin B_{12} Absorption**

VITAMIN B_{12} is the only known nutrient for which there is required a specific gastric secretion to promote its absorption. It is not known whether the intrinsic factor is absorbed with vitamin B_{12}. The predominant site of absorption in man is believed to be the ileum. Without this gastric secretion persons will become deficient in vitamin B_{12} in spite of a "normal" dietary intake. Vitamin B_{12} which has been biosynthesized with radioactive cobalt as an integral part of the molecule has greatly facilitated the study of vitamin B_{12} absorption in health and in a variety of diseases.

Absorption may be estimated from radioactivity in the feces, urine, plasma, or over the liver. The percentage of the oral dose which is absorbed decreases rapidly as the oral dose is increased above one or two micrograms. To have any meaning, the figure "percentage of the oral dose absorbed (or excreted)" must be stated in reference to the quantity of vitamin B_{12} in the oral dose, and the range of normal will vary depending upon this quantity. To illustrate the importance of the quantity of vitamin B_{12} ingested, one should compare the average percentage of orally administered radioactivity which appears in the urine after a 0.5 μg dose with that appearing after a 2.0 μg dose: 26 per cent vs. 11 per cent. It should be evident that the physiologic significance revolves around the quantity of the vitamin absorbed, *not* the quantity of radioactivity.

Vitamin B_{12} which is bound to intrinsic factor appears to be absorbed preferentially over the unbound vitamin given at the same time. Therefore, one must be certain that the intrinsic factor preparation being tested by radioactive-B_{12} technics is free of nonradioactive vitamin B_{12}. Because of the variability of patients with pernicious anemia, one must be wary of the comparison of intrinsic factor preparation A in patient A with intrinsic factor preparation B in patient B. It is better to compare preparations A and B in the same patient.

Recent publications have described the finding that several hog intrinsic factor preparations, which were known to be active as sources of intrinsic factor in patients with pernicious anemia, inhibited the absorption of a single dose of vitamin B_{12} by some normal persons. There is insufficient evidence to conclude that intrinsic factor preparations should be administered to persons who have normal gastric function. The available data demonstrate that this "inhibition" of vitamin B_{12} absorption in normals is probably due to hog or partially denatured intrinsic factor.

Published data have demonstrated that hog intrinsic factor preparations given daily with vitamin B_{12} over a period of months will raise the serum vitamin B_{12} levels of "normal" or elderly recipients. Of interest are the several reports of treatment of pernicious anemia with oral intrinsic factor-vitamin B_{12} combinations over a period of many months. The hematologic relapse rate has been unexpectedly great and the serum vitamin B_{12} levels have not risen to normal. It would appear that pernicious anemia and other forms of vitamin B_{12}-deficiency due to malabsorption of the vitamin should be treated by injections of the vitamin.

* This editorial has been prepared at the request of the Editorial Board.

After the oral ingestion of "physiologic" (i.e., 1–2 μg) doses of radioactive vitamin B_{12}, there is a delay of three to four hours before radioactivity appears in the plasma, and the maximum is reached at about eight to twelve hours. If a much larger oral dose of vitamin B_{12} is given, radioactivity will appear in the plasma much sooner. This second mechanism for absorbing at higher oral doses appears to be intact in patients with pernicious anemia.

Sorbitol (D-sorbose) has been shown to enhance plasma vitamin B_{12} levels in normal subjects given oral doses of the vitamin. It is possible that this action is due to a stimulation of gastric secretion of intrinsic factor. Data showing the effects of D-sorbose on vitamin B_{12} absorption in patients unable to produce intrinsic factor will be helpful in understanding the mechanism of action of this compound in normals.

The above comments demonstrate a well-known truism in biology: the answers to yesterday's questions provide the framework for today's problems.[6]

—ROBERT F. SCHILLING, M.D.
University of Wisconsin Medical School
Madison, Wisconsin

Letter to the Editor

ON ENHANCEMENT OF VITAMIN B_{12} ABSORPTION

Dear Sir:

The demonstration by Chow, Meier, and Free[1] of facilitation of enteral vitamin B_{12} absorption by admixture with D-sorbitol is of extreme interest, but this may not have been "the first substance other than intrinsic factor" shown to effect such absorption. Some time previously I reported in another connection[2] that on administration by mouth of crude, streptomyces-derived vitamin B_{12} concentrates emulsified in vegetable oil with sorbitan monooleate polyoxyethylene derivative (Tween 80) there appeared to be marked vitamin B_{12} assimilation as evidenced by the same pinkish "skin-flush" and copious diuresis which had been noticed in an occasional patient receiving large parenteral doses of cobalamine. Since the patients in the particular series reported were not those with Addisonian anemia (and therefore may have had absorption conditioned by intrinsic factor) another study was conducted with three patients with relapsant pernicious anemia.

It had been determined previously that hydroxycobalamine, cyanocobalamine, nitrocobalamine, and carbimidocobalamine were all soluble in oleic acid-glycerol trioleate mixtures and that such solutions immediately dispersed to less than 0.5 micron droplet size in aqueous menstruums when 2 to 20 per cent of Tween 80 was incorporated. A single, one ml dose of such Tween-oil-oleic acid solutions containing 500 μg of hydroxycobalamine was given orally to each of three patients with pernicious anemia in relapse.

However, because high oral dosing with ordinary vitamin B_{12} preparations was already known to be followed by remission of classic pernicious anemia, this eventuation in all three patients was not used as a criterion for absorption. Instead, total 24-hour urine outputs, both immediately before and after the test dosings were given to Dr. Roger Kersey of Chas. Pfizer and Company for vitamin B_{12} assay. These assays on the post-dosing specimens were rendered difficult because of unanticipated *high* vitamin B_{12} content which necessitated repeated dilutions to get within the range of the assay procedure.

It was finally determined that each patient excreted from 50 to 150 μg of vitamin B_{12} (activity) during the 24 hours following the 500 μg dose, an order of magnitude, which while not approaching that which would have followed

the parenteral administration of 500 μg of vitamin B_{12}, was certainly much greater than could be accounted for on the basis of intrinsic factor carriage.

I had speculated (in the publication cited above) that the enteral absorption of vitamin B_{12} in Tween-oil solution might be conditioned by those same factors which operated in facilitation by Tween of enteral absorption of any "fat-soluble" vitamin, which vitamin B_{12} becomes in this instance. Such facilitation is usually explained by circumvention of the portal circulation through the regular "fat-droplet" route into the lacteal villi and thence to the thoracic duct. There is as yet no good reason for abandoning this explanation although the thesis posed by Chow, Meier, and Free may offer an alternative one for the effects I observed. D-sorbitol is a constituent of the polysorbate macromolecule. Perhaps even as such it could effect transintestinal vitamin B_{12} transport. Polysorbate is hydrolyzed to some extent by pancreatic lipase;[3] one of its products could be D-sorbitol.

Lipases, like all other enzymes, have synthetic as well as degradative actions and it occurred to Gordon that intestinal synthesis of "tweens" might take place if suitable hexitols were fed. It was known that a crude, dried streptomycin beer, naturally rich in D-mannitol was effective when given orally in megaloblastic anemia.[4] He and his co-workers reported similar effectiveness of a vitamin B_{12} sweetened with D-mannitol[5] at about the same time as D-mannitol was found by Greenberg's group to have the same qualitative efficacy in facilitating vitamin B_{12} absorption by the intestinal tract of the rat, as does D-sorbitol.

—ROBERT D. BARNARD, M.D.
Division of Surgical Research,
Harlem Hospital,
New York City Department
of Hospitals.

REFERENCES

1. CHOW, B. F., MEIER, P., and FREE, S. M : Absorption of vitamin B_{12} enhanced by D-sorbitol. AM. J. CLIN. NUTRITION 6:30, 1958.
2. BARNARD, R. D.: The effect of streptomyces fermentation residue on "hemolytic" anemia with notes on some similarities of the effects of intestinal flora reversion and those of adrenocorticotherapy. *Int. Rec. Med.* 164:117, 1951.
3. CULVER, P. J., WILCOX, C. S., JONES, C. M., and ROSE, R. S., JR.: Intermediary metabolism of certain polyoxyethylene derivatives in man: recovery of polyoxyethylene moiety from urine and feces following ingestion of (20) sorbitan monooleate and (40) monostearate. *J. Pharmacol. & Exper. Therap.* 103:377, 1951.
4. BARNARD, R. D., CARACAPPA, J. M., SCHWARTZ, M., GORDON, G. B., and MOLDOVER, A.: Further observations on the response of erythroblastic, megaloblastic and radiation anemias to streptomyces-derived "animal protein factor" and antibiotics, *New York J. Med.* 51:1739, 1951.
5. GORDON, G. B., RUBIN, H., STEIN, S., and KESSLER, L. N.: The effect of D-mannitol incorporation on vitamin B_{12} dosage forms. Oncology Research Conference, Jewish Chronic Diseases Hospital, Brooklyn, January, 1958.
6. GREENBERG, S. M., HERNDON, J. F., RICE, E. G., PARMELEE, E. T., GULESICH, J. J., and VAN LOON, E. J.: Enhancement of vitamin B_{12} absorption by substances other than intrinsic factor. *Nature* 180:1401, 1957.